INVESTIGATION OF INHIBITORS OF POLYSIALYLTRANSFERASE AS NOVEL THERAPEUTICS FOR NEUROBLASTOMA

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Development of *in vitro* assays to assess the functionality and selectivity of novel small-molecule inhibitors of polysialyltransferases for use in neuroblastoma therapy

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Abstract

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Investigation of inhibitors of polysialyltransferase as novel therapeutics for neuroblastoma

Keywords: *Polysialic acid, Neural Cell Adhesion Molecule, Neuroblastoma, Polysialyltransferase, Metastasis, Invasion, Cancer therapeutics, Transwell invasion assay, Sialic acid expression*

Polysialic acid is a unique carbohydrate that decorates the surface of the neural cell adhesion molecule. Polysialic acid is an onco-developmental antigen, expressed in tumours principally of neuroendocrine origin, notably neuroblastoma, strongly correlating with invasion and metastasis. Polysialylation is regulated by two polysialyltransferase enzymes, PST (ST8SiaIV) and STX (ST8SiaII), with STX dominant in cancer. Post-development polysialic acid expression is only found at low levels in the brain, thus this could be a novel target for cancer therapy. It is hypothesized that inhibition of polysialyltransferase could lead to control of tumour dissemination and metastasis. The aims of this thesis were to develop tools and *in vitro* assays to screen novel polysialyltransferase inhibitors. A panel of tumour cell lines were characterised in terms of growth parameters (using the MTT assay) and polysialic acid expression. This includes a pair of isogenic C6 rat glioma cells (C6-STX and C6-WT) and naturally polysialic acid expressing neuroblastoma cells (SH-SY5Y). Following this, an *in vitro* assay was validated to screen modulation of polysialic acid expression by removing pre-existing polysialic acid expression using endoneuraminidase N and evaluated the amount of re-expression of polysialic acid using immunocytochemistry. Then, a functional assay was developed and validated for invasion, the matrigel invasion assay. Cytidine monophosphate (tool compound) significantly reduced polysialic acid surface expression and invasion. A panel of six novel polysialyltransferase inhibitors was screened for cytotoxicity, polysialic acid surface expression and invasion. Of the potential polysialyltransferase inhibitors evaluated, ICT3176 and ICT3172 were identified from virtual screening of Maybridge library and were emerged as the most promising inhibitors, demonstrating significant ($p<0.05$) reduction in cell-surface polysialic acid re-expression and invasion in polysialic acid expressing cells. Furthermore, the specificity of compounds for polysialyltransferase (α-2,8-sialyltransferase) over other members of the wider sialyltransferase family (α-2,3- and α-2,6-sialyltransferases) was confirmed using differential lectin staining. These results demonstrated that small molecule inhibitors as STX is possible and provides suitable *in vitro* cell based assays to discovery more potent derivatives.

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Abbreviations

Abbreviation	Explanation
ADCC	Antibody-dependent cellular cytotoxicity
ADME	Absorption Distribution Metabolism and Excretion
ATCC	American Type Culture Collection
BME	Basement membrane extract
CD24	Mucin type glycoprotein
CDC	Complement-dependent cytotoxicity
Ch	Chimeric
CMP	Cytidine monophosphate
CT	Computed tomography
CYP	Cytochrome P450
DAPI	4,6-Diamidino-2-phenylindole
DMSO	Dimethyl sulphoxide
ECM	Extracellular matrix
EDTA	Ethylenediamine tetraacetic acid

EHS	Engelbreth Holm Swarm
Endo-N	Endoneuraminidase-N
EndoN-GFP	Endoneuraminidase-N green fluorescent protein
ERK	Extracellular signal-regulated kinase
FAK	Focal adhesion kinase
FBS	Fetal bovine serum
FGF	Fibroblast growth factor
FGFR	Fibroblast growth factor receptor
FITC	Fluorescein isothiocyanate
FnIII	Fibronectin-type III repeats
Gal	Galactose
GBM	Glioblastoma
GD2	Ganglioside
GM-CSF	Granulocyte-macrophage colony stimulating factor
GPI	Glycosylphosphatidylinositol
HBSS	Hank's balanced salt solution

HTC	High throughput screening
HVA	Homovanillic acid
IC_{50}	Inhibitory concentration
ICC	Immunocytochemistry
ICD-O	International Classification of Diseases for Oncology
ICT	Institute of Cancer Therapeutics
Ig	Immunoglobulin
IgG	Immunoglobulin g
IHC	Immunohistochemistry
INRG	International Neuroblastoma Risk Group
INSS	International Neuroblastoma Staging System
LA	Lithocholic acid
MAL I	*Maackia amurensis* leukoagglutinin
MAL II	*Maackia amurensis* hemagglutinin
ManNAc	*N*-acetylmannosamine
ManNProp	*N*-propanoyl mannosamine

MIBG	Meta-iodobenylguanidine
MRD	Minimal residual disease
MTD	Maximum tolerance dose
MTT	3-(4,5-Dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide
MYCN	V-myc myelocytomatosis viral related oncogene, neuroblastoma derived
NCAM	Neural cell adhesion molecule
NHS	Normal horse serum
NRS	Normal rabbit serum
NSCLC	Non-small cell lung cancer
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PD	Pharmacodynamics
PFA	Paraformaldehyde
PK	Pharmacokinetics
PolySia	Polysialic acid

PolySia-NCAM	Polysialylated NCAM
PolyST	Polysialyltransferase
PolySTi	Polysialyltransferase inhibitor
PST	Polysialyltransferase IV
PSTD	Polysialyltransferase domain
RA	Retinoic acid
RNA	Ribonucleic acid
RT-PCR	Reverse transcription-polymerase chain reaction
s.c	Subcutaneous
SCID	Severe combined immunodeficiency
SCLC	Small cell lung cancer
Sia	Sialic acid
siRNA	Small interfering RNA
SNA	Sambucus nigra agglutinin
ST	Sialyltransferase
STX	Polysialyltransferases II

SVZ	Subventricular zone
TRITC	Tetramethylrhodamin isothiocyanate
VPA	Valproic acid
VMDA	Vanillylmandelic acid
WT	Wild type

CHAPTER ONE

1 Introduction

1.1 Aetiology of Cancer

Cancer is a multi-faceted disease characterised by uncontrollable cell proliferation (Hanahan and Weinberg, 2011). There have been more than 200 different types of cancer identified, caused by random mutations of genes or exposure to environmental changes (Hanahan and Weinberg, 2000). 161,823 deaths in the UK were due to cancer in 2012, of which half were from lung, bowel, breast and prostate cancers (CRUK, 2014).

Cancer is rare among children, affecting between 70 and 160 per million children 0-14 years (Stiller, 2004). There are histological differences between adult and childhood cancer, and therefore cancers been classified according to

histology rather than a primary site (Stiller, 2004). The 'International Classification of Childhood Cancer' includes 12 major groups sub-divided into 47 sub-groups. These are based on morphology and topography as stipulated in the third edition of the International Classification of Diseases for Oncology (ICD-O) (Steliarova-Foucher et al., 2005).

These cancers have different origins but their characteristic biological ability to promote tumour growth and their metastatic dissemination provides the fundamental foundation, which helps to understand the complex biology of cancers.

The major groups of childhood cancers include leukaemia, lymphomas, brain and spinal tumours. It also includes sympathetic nervous system tumours, retinoblastoma, kidney tumours, hepatic tumours, malignant bone tumours, soft tissue sarcomas, gonadal and germ-cell tumours, epithelial tumours, as well as other and unspecified malignant cancers (Stiller, 2004). The most common extra-cranial cancer in children which is also the second-most common paediatric cancer is neuroblastoma (Park et al., 2010).

1.2 Neuroblastoma

Neuroblastoma is a paediatric cancer affecting the sympathetic nervous system (Maris et al., 2007). Neuroblastoma is the most deadly solid tumour in children causing 7 to 10% of paediatric oncology deaths. Many cases of neuroblastoma occur in children either during infancy or early childhood (Brodeur, 2003). The

incidence of neuroblastoma is relatively slightly higher in boys than in girls (Heck et al., 2009).

1.2.1 Clinical presentation

Most neuroblastoma is characterised by the presence of undifferentiated tumour cells called neuroblasts (Brodeur, 2003). Neuroblastoma arises from neural crest cells, which are progenitor cells of the sympathetic nerves. These migrate from the neural crest and from sympathetic ganglia, the chromaffin cells of the adrenal medulla and the paraganglia (Park et al., 2010).

The symptoms and clinical presentation of neuroblastoma patients are a common indication of tumour location (Ishola and Chung, 2007). The clinical presentation reflects the primary tumour location and the intensity of the disease (Weinstein et al., 2003). 65% of primary tumours are found in the abdomen (adrenal glands) and other common sites are the neck, chest and pelvis (Maris et al., 2007).

The clinical staging classification includes localised tumours and those which have metastasised are described in section 1.2.2. Localised tumours occur in 40% of patients and range in diagnosis of an intra-adrenal mass to large invasive tumours along with the sympathetic chains (Maris et al., 2007). The intra-abdominal mass may be an incidental finding on ultrasonography. There are approximately 5% patients who suffer from the signs of neurological impairment which includes motor weakness, pain or sensory loss.

Neuroblastoma is a metastatic disease which burdens the system extensively. Patients with localised disease show no symptoms but patients with metastatic disease have symptoms, namely fever and bone pain often caused by tumour dissemination (Weinstein et al., 2003).

1.2.2 Classification

Clinically, there are three patterns of neuroblastoma which include rapid progression of the tumour into a life-threatening illness, maturation to benign ganglioneuroma and spontaneous regression (Heck et al., 2009). The progression and typology of neuroblastoma, like many other cancers, is defined by stages. In 1984, Shimada and colleagues described the staging classification relating to tumour (histological features or biopsy) which has shown high levels of urinary catecholamines as listed in **Table 1.1** (Shimada et al., 1984).

Stage 1 is localised tumours that can be completely resected whereas stage 2 and stage 3 involve tumours which can be partially resected with or without regional nodules. These stages are dependent on the potential for removing the volume of tumour resectable, the local invasion and lymph nodes.

Stage 4 can be defined as dissemination of tumours to distant lymph nodes or bone marrow. The striking phenotypes of neuroblastoma in stage 4S (S for special) was first described in 1971 (D'Angio et al., 1971). In infants (stage 4S) is defined as the dissemination patterns limited to liver, skin and minimal bone marrow. Spontaneous regression is the most enigmatic clinical feature of neuroblastoma and is associated with stage 4S. Spontaneous regression of 4S

tumours is reported with myelocytomatosis viral related oncogene, neuroblastoma derived (*MYCN)* amplification (Tonini et al., 1997) or lack of *MYCN* amplification Stage 4S tumour (Nickerson et al., 2000).

Table 1.1: Schematic illustration of the International Neuroblastoma Staging System (INSS). Taken from (Subhasree Roy Choudhury, 2012).

International Neuroblastoma Staging System	
Stage 1	Localised tumour with complete gross excision; with/out microscopic residual disease; identifiable ipsilateral and contralateral lymph nodes is negative microscopically
Stage 2A	Localised tumour with incomplete gross excision; representative ipsilateral non-adherent regional lymph nodes negative for tumour microscopically
Stage 2B	Localised tumour with/out complete gross excision; with ipsilateral non-adherent lymph nodes; positive for tumour; enlarged contralateral lymph nodes must be negative microscopically
Stage 3	Unresectable unilateral tumour infiltrating across the midline; with/out regional lymph nodes involvement; localised unilateral tumour with contralateral regional lymph nodes; midline tumour with bilateral extension by unresectable (infiltration) or by lymph nodes involvement
Stage 4	Primary tumour (any) dissemination to distant lymph nodes, bone marrow, skin or other organs
Stage 4S	Localised primary tumour (defined for stage 1, 2A, 2B) with dissemination limited to skin, liver or bone marrow (limited to infants<1 year of age)

1.2.3 Prognostic factors

The International Neuroblastoma Surgical Staging (INSS) refers to the surgically based staging systems and has limitations including: staging patients are found more in high-neuroblastoma treatment and overlapping types of treatment. Due to these limitations, the International Neuroblastoma Risk Group (INRG) was developed in 2005, based on the clinical and biological features of 11,054 neuroblastoma patients across the world. The list includes age at diagnosis, INSS stage, tumour histopathology, DNA index (ploidy) and *MYCN* amplification, which are considered to be significant risk factors for neuroblastoma (Cohn et al., 2009, Park et al., 2010). These clinical and biological prognosis factors are statistically significant and clinically relevant for prognostic evaluation (**Table 1.2**) (Cohn et al., 2009).

Neuroblastoma prognosis is dependent on age and location of the tumour, but the most important prognostic factor is the stage of the disease (Weinstein et al., 2003). Age is also an important prognostic factor and there is a worse prognosis for patients older than 1 or 2 years, especially when diagnosing disseminated disease (Park et al., 2010). Heck et al (2009) identified 47 articles from the search engine PubMed and summarised the risk factors in pregnancy for the later development of neuroblastoma in children. These included use of alcohol and effects of intake of vitamin during pregnancy or intake leading to allergic reactions prior to the diagnosis of neuroblastoma (Heck et al., 2009). These studies provide a complicated understanding of the causation of neuroblastoma and further studies are still required.

Table 1.2: Children Neuroblastoma Risk Group (INRG) based on clinical features. Taken from (Park et al., 2010).

Stage	Risk group	Age (months)	Ploidy	*MYCN*	Shimada
1	Low risk				
2A/2B	Low risk			NA	
2A/2B	High risk			A	
3	Intermediate risk	<18		NA	
3	Intermediate risk	≥18		NA	FH
3	High risk			A	
3	High risk	≥8		NA	UH
4	High risk	<12		A	
4	Intermediate risk	<12		NA	
4	High risk	12 to < 18		A	
4	High risk	12 to < 18	DI = 1		
4	High risk	12 to < 18			UH
4	Intermediate risk	12 to < 18	DI > 1	NA	FH
4	High risk	≥18			
4S	Low risk	<12	DI > 1	NA	FH
4S	Intermediate risk	<12	DI = 1	NA	
4S	Intermediate risk	<12		NA	UH
4S	High risk	<12		A	

Keywords: Blank: Any field; DI: DNA Index; FH: Favourable Histology; UH: Unfavourable Histology; A: Amplified; NA: Not Amplified; Shimada histopathologic classification

1.2.4 Diagnostic tests

Different types of visual and physical symptoms and screening tests are used for the diagnosis of neuroblastoma. This includes biopsy, testing for urine or serum metabolites (catecholamine), metastatic spread, and size of tumour monitored by computed tomography and magnetic resonance imaging (Maris et al., 2007, Park et al., 2010).

Chest radiographs allow tumour detection whereas ultrasonography allows determination of the nature of the tumour (Ishola and Chung, 2007). Computed tomography (CT) can also be used for the clinical assessment of the disease in the abdomen, pelvis or mediastinum regions (Maris et al., 2007). Nearly 80% of neuroblastoma patients produce urinary catecholamines. The metabolites of catecholamine; homovanillic acid (HVA) and vanillylmandelic acid (VMDA) are assessed for the diagnostic screening of the disease (LaBrosse et al., 1980, Laug et al., 1978).

Neuroblastoma is often a radiation sensitive disease and there has been considerable interest in using radioactive isotope-labelled, which are taken by tumours, particularly meta-iodobenzylguanidine (MIBG). This is a norepinephrine analogue taken up by high catecholamine excreting organs. Therefore, radiolabelled 131-I MIBG allows imaging of neuroblastoma and could have a potential for therapy in high-risk neuroblastoma patients (Riad et al., 2009).

1.2.5 Treatments

Presently, various therapeutic options are available for neuroblastoma, which depend on the patient's age and stage of the disease. Treatment of patients with stage 4 disease remains the biggest challenge for paediatric oncologists. Currently, the treatment options consist of chemotherapy, radiotherapy or local control with surgery.

1.2.5.1 Surgery

The general surgical approach is used for resection, staging of tumour and biopsy. Different surgical procedures are required for different stages of neuroblastoma, which are described in **Table 1.2**. In the early stages of neuroblastoma (stages 1, 2A, 2B) surgery resection is preferred option. This may be used in 4S stages since tumour may be differentiate and spontaneously regress without any specific treatment (Ishola and Chung, 2007).

1.2.5.2 Chemotherapy

Chemotherapy is mainly used by the patients who have relapsed after surgery in INSS stage 1. The typical chemotherapeutic agents used are cyclophosphamide, iphosphamide, vincristine, doxorubicin (adriamycin), cisplatin, carboplatin, etoposide (VP-16) and melphalan that are used in different combinations (Ishola and Chung, 2007). For example, myeloablative chemotherapy has been used as a combined therapy with 131-I MIGB for

treating INSS 3 and INSS 4 stage patients with refractory neuroblastoma (Matthay et al., 2006).

In the advanced stage tumours, combined chemotherapy reduced the tumour size, which can be resectable, but often failed to eradicate the tumour. Some cases of high-risk neuroblastoma patients (INSS 3 and INSS 4), where neuroblastoma is refractory to chemotherapy are aggressively treated by bone marrow-ablative therapy with melphalan or total body radiation followed by bone marrow transplant, which have shown some success (Matthay et al., 2006). However, these therapies have unfavourable side-effects.

1.2.5.3 Radiotherapy

Neuroblastoma is considered to be radiosensitive tumour and radiotherapy has shown to be applied with minimal residual disease (MRD) therapy and surgery (Modak and Cheung, 2010). Radiotherapy and chemotherapy are often used together for improving the resectability of advanced stage neuroblastoma (Ishola and Chung, 2007).

1.2.5.4 Myeloablative therapy

Several clinical trials have shown that myeloablative therapy combined with bone marrow transplant improve high-risk patients' neuroblastoma outcomes. With this therapy, the relapse rate can be reduced in neuroblastoma and further intensification of myeloablative therapy may result in better outcomes (Park et al., 2010).

1.2.5.5 Other new therapies

Recently, new therapies have also been developed and are having a huge impact on the clinical treatments of neuroblastoma. The most common biotherapy adjuvant is the 13-*cis*-retinoic acid (isotretinoin, 13-*cis*-RA), a synthetic retinoid that is used in treating high-risk neuroblastoma (Park et al., 2010).

New therapeutic agents for neuroblastoma, including topotecan (topoisomerase I inhibitor) or combinational therapy with carboplatin have a potential use against neuroblastoma for relapse patients (Weinstein et al., 2003). Other new approaches include using immunotherapy (targeting disialoganglioside GD2) or angiogenesis inhibitors (Maris et al., 2007).

Despite the availability of various treatments and diagnostic tools, neuroblastoma still continues to have a poor prognosis rate. The majority of neuroblastoma patients are younger than a year old, and the majority die as the disease progresses. Less than 40% of children survive up to 5 years (Subhasree Roy Choudhury, 2012). This emphasizes the urgent need for developing new therapeutic strategies to treat neuroblastoma.

Hanahan and Weinberg (2000) proposed the 'Hallmarks of the Cancer' which consist of characteristics relating to a biological ability to promote tumour growth and tumour dissemination, providing us the fundamental basis to our understanding of cancer. This research is primarily focused on inhibiting invasion and metastasis.

The dissemination of tumour cells from primary lesion sites is the main cause of cancer mortality and morbidity. Targeting this hallmark could provide a novel therapeutic opportunity. Good anti-metastatic drugs could be developed through understanding the biology of the tumour and different molecules playing a role in tumour progression.

One novel target is aberrant glycosylation in neoplastic tissues, especially abnormal sialylation expression, which is a particular feature of the malignant tumour, affecting the adhesive interactions of cancer cells and provides favourable conditions for tumour dissemination (Rambaruth and Dwek, 2011).

1.3 Tumour glycocalyx

Tumour progression and metastasis is a multi-step process. An important process in the metastatic cascade is the role of cell adhesion molecules involved in protein-glycan interactions reviewed in Rambaruth and Dwek (2011). Glycans play a role in cell-adhesion, cell-cell and cell-matrix communication, and in detachment from the extracellular matrix and cell mobility for invasion (Peracaula et al., 2008). Some aberrant glycans have been reported in tumour cells assisting in tumour development and progression (Fuster and Esko, 2005).

Various carbohydrates decorate the surface of mammalian cells (**Figure 1.1**), collectively known as the glycocalyx and individually as ‘glycans’ which involve the formation of the oligo- and polysaccharides from monosaccharides (Yarema and Bertozzi, 2001). Glycans can be classified either as *N*-linked forming a glycosidic bond with asparagine or *O*-linked which form a glycosidic bond with

serine or threonine. They can be branched and regularly terminate with a single sugar unit belonging to the sialic acid family, playing a diverse functional role in cell-cell interaction (Fuster and Esko, 2005).

The aberrant expression of glycocalyx components has been linked to tumour progression. One key component of the glycocalyx is polysialic acid (polySia), which was first described by Finne in 1982 as a major component of developing vertebrate brains. PolySia is a carbohydrate polymer of sialic acid predominantly attached to the neural cell adhesion molecule (NCAM) (Fukuda, 1996, Kiss and Rougon, 1997).

Many studies have indicated the expression of modified NCAM by polySia in cancers including neuroblastoma, rhabdomyosarcoma, Wilm's tumour, small-cell lung and non-small cell lung cancer (Fukuda, 1996, Glüer et al., 1998a), where it correlates with tumour progression and metastasis (Cheung et al., 2006, Daniel et al., 2001, Gluer et al., 1998c, Tanaka et al., 2000). Since, there is a positive correlation between polysialylated NCAM and cancer metastasis, developing an anti-metastatic drug against aberrant polySia-NCAM could hold a promising future.

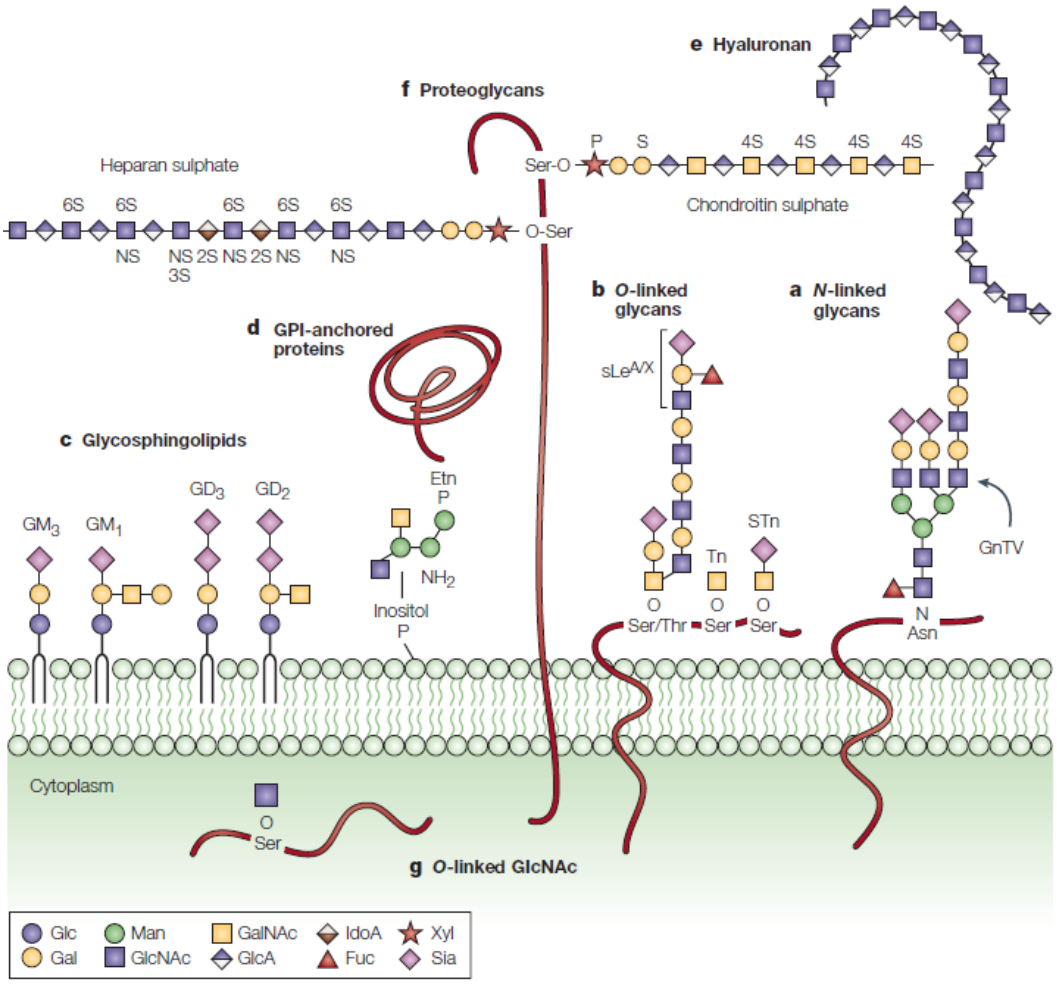


Figure 1.1: Unique glycans involved in tumour progression.

Glycoproteins either branched with (a) *N*-linked glycans (conjugated bond to asparagine) or with (b) *O*-linked glycans (covalently linked to serine or threonine). (c) Glycosphingolipids, which consist of glycans linked to ceramide. Glycosaminoglycans can either occur as free chain (hyaluronan: (e) or covalently linked with proteoglycan core protein (heparan sulphate: (f). Coloured geometric symbols are used to represent various sugars. Taken from (Fuster and Esko, 2005).

Keywords: Glc: Glucose; Gal: Galactose; Man: Mannose; GlcNAc: *N*-acetylglucosamine; GalNAc: *N*-acetylgalactosamine; GlcA: Glucuronic acid; Fuc: Fucose; Xyl: Xylose; Sia: Sialic acid.

1.4 Neural cell adhesion molecule

Nearly 40 years ago, the neural cell adhesion molecule (abbreviated as NCAM; CD56) was identified as a cell surface glycoprotein (Rutishauser et al., 1976). In subsequent years, NCAM was isolated, purified and fully characterised where it was shown to be acting as an adhesion mediator in retina cells (Hoffman et al., 1982, Thiery et al., 1977). NCAM belongs to the immunoglobulin superfamily, which is predominantly present in the central and peripheral nervous system (Angata and Fukuda, 2003, Jensen and Berthold, 2007).

NCAM is encoded by a single gene and is found on chromosome 11 in humans and on chromosome 9 in mice (Nguyen et al., 1986). Alternative RNA splicing of NCAM results in three major isoforms, namely NCAM-180, NCAM-140 and NCAM-120 (**Figure 1.2**) (Gascon et al., 2007). These isoforms have different C-terminal regions: the NCAM-120 isoform, connected by a glycosylphosphatidylinositol (GPI) anchor to the cell membrane, is predominantly expressed in normal and well differentiated tissues. The NCAM-140 and NCAM-180 isoforms have transmembrane domains and are found predominantly in less differentiated or malignant cell types (Jensen and Berthold, 2007). NCAM isoforms have identical N-terminal extracellular regions consisting of five immunoglobulin-like (Ig1-5) domains and two fibronectin type III domains (Jensen and Berthold, 2007, Bonfanti, 2006). Furthermore, these isoforms are expressed in different cell types, for instance, NCAM-120 is expressed in glial, and NCAM-140 in neuron and glial and NCAM-180 is found in neurons (Noble et al., 1985).

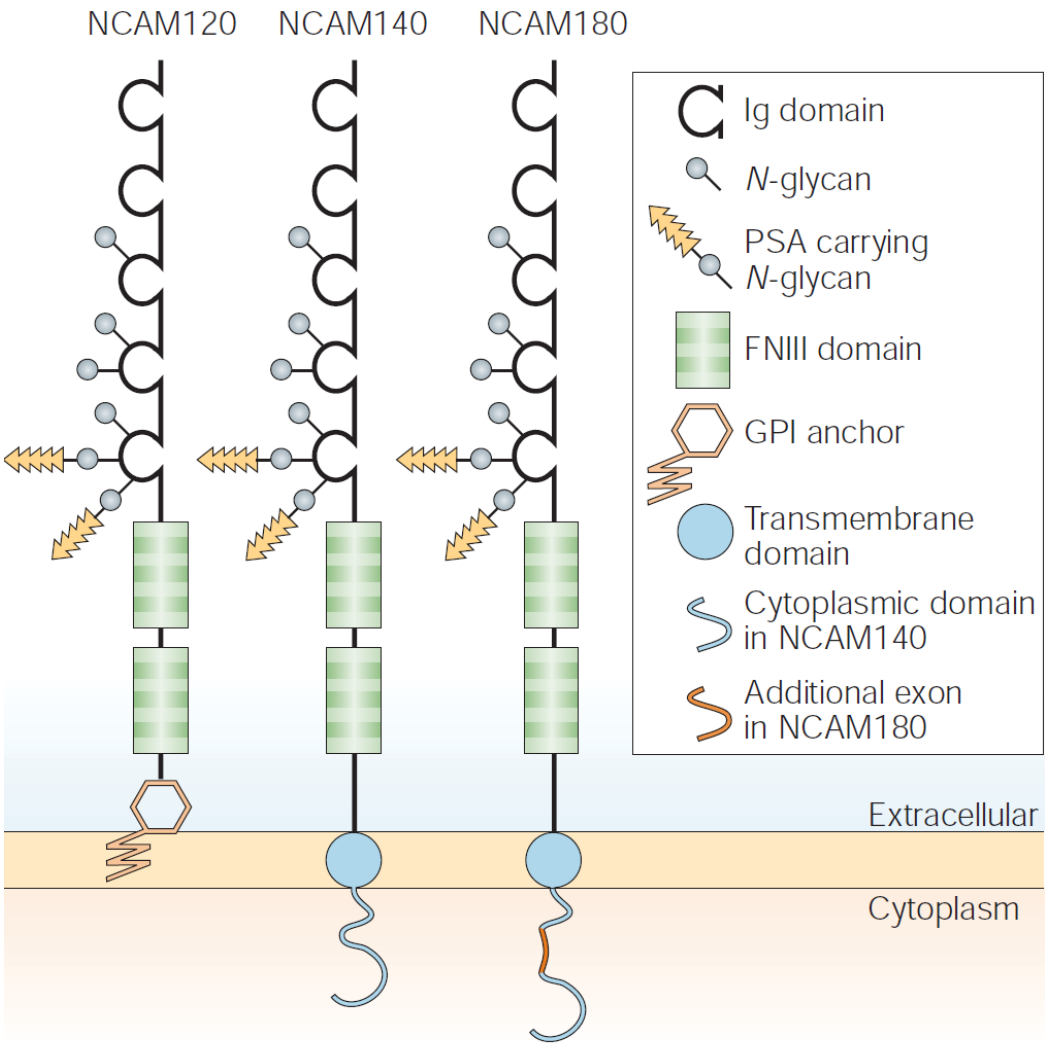


Figure 1.2: Schematic illustration of molecular characteristics of NCAM.

The NCAM protein core is attached to polySia via two *N*-glycosylation sites, containing five immunoglobulin-like domains (Ig) and two fibronectin-type III repeats (FnIII). NCAM isoforms NCAM-180 and NCAM-140 are transmembrane protein whereas NCAM-120 linked by GPI anchor result by an alternative splicing. (Kleene and Schachner, 2004).

1.5 Sialic acid

The wider sialic acid family encompasses approximately fifty naturally occurring sugars, the most common of which is *N*-acetylneuraminic acid ('sialic acid': Sia) found in humans (Kohla and Schauer, 2005). Sia is a negatively charged nine-carbon sugar, commonly found on the cell-surface, at glycan termini. Depending on the chemical linkage, Sia forms *O*- or *N*-linked glycans, attached either to galactose (Gal) or *N*-acetylgalactosamine (GalNAc) via α-2,3-/α-2,6- linkages or α-2,8/α-2,9-linkages in the case of polysialic acid by specific enzymatic reactions (Ivan Martinez-Duncker, 2011). Therefore, any changes in glycan expression such as enzyme dysfunction activity can lead to rare congenital diseases or cancer (Galeano et al., 2007, Li et al., 2010).

1.6 Polysialic acid

Polysialic acid (polySia) is composed of a unique carbohydrate, which is a linear homopolymer of α-2,8-linked sialic acid expressed predominantly in the embryonic brain (Brusés and Rutishauser, 2001, Finne, 1982, Bonfanti, 2006). A post-translational process adds polySia on NCAM, forming simple and large molecules and has a range from 8 to 100 monomers (Mühlenhoff et al., 1998, Rutishauser and Landmesser, 1996).

PolySia has three main building units: 5-*N*-acetylneuraminic acid (Sia), 5-*N*-glycolylneuraminic acid (Neu5Gc) and 5-deamino-3, 5-deoxyneuraminic acid (2-

keto-3-deoxynonulosonic acid, Kdn) (Mühlenhoff et al., 1998) shown in **Figure 1.3A**.

In mammals, the majority of polySia is expressed on NCAM (see **Figure 1.3B**). However, polySia expression has been detected on the voltage-sensitive sodium channel alpha-unit and secreted glycoprotein in mammalian milk CD36 (Zuber et al., 1992, Yabe et al., 2003). In addition to this, polysialylated NCAM has been found in other cells including oligodendrocytes, astrocytes, neuroblasts, glial precursors and on the immune system including human leukocyte to modulate immune response (for details, see (Janas and Janas, 2011, Drake et al., 2008)).

1.6.1 Biological and structural features of polySia

The carboxyl region of Sia has been used to determine the biological features of polySia, consisting of a negative charge, covering a large space and sustaining water and ionic molecules (Gascon et al., 2007, Kiss and Rougon, 1997, Rutishauser and Landmesser, 1996). PolySia attenuates NCAM interactions, modulating cell adhesion properties (Rutishauser and Landmesser, 1996, Kiss and Rougon, 1997).

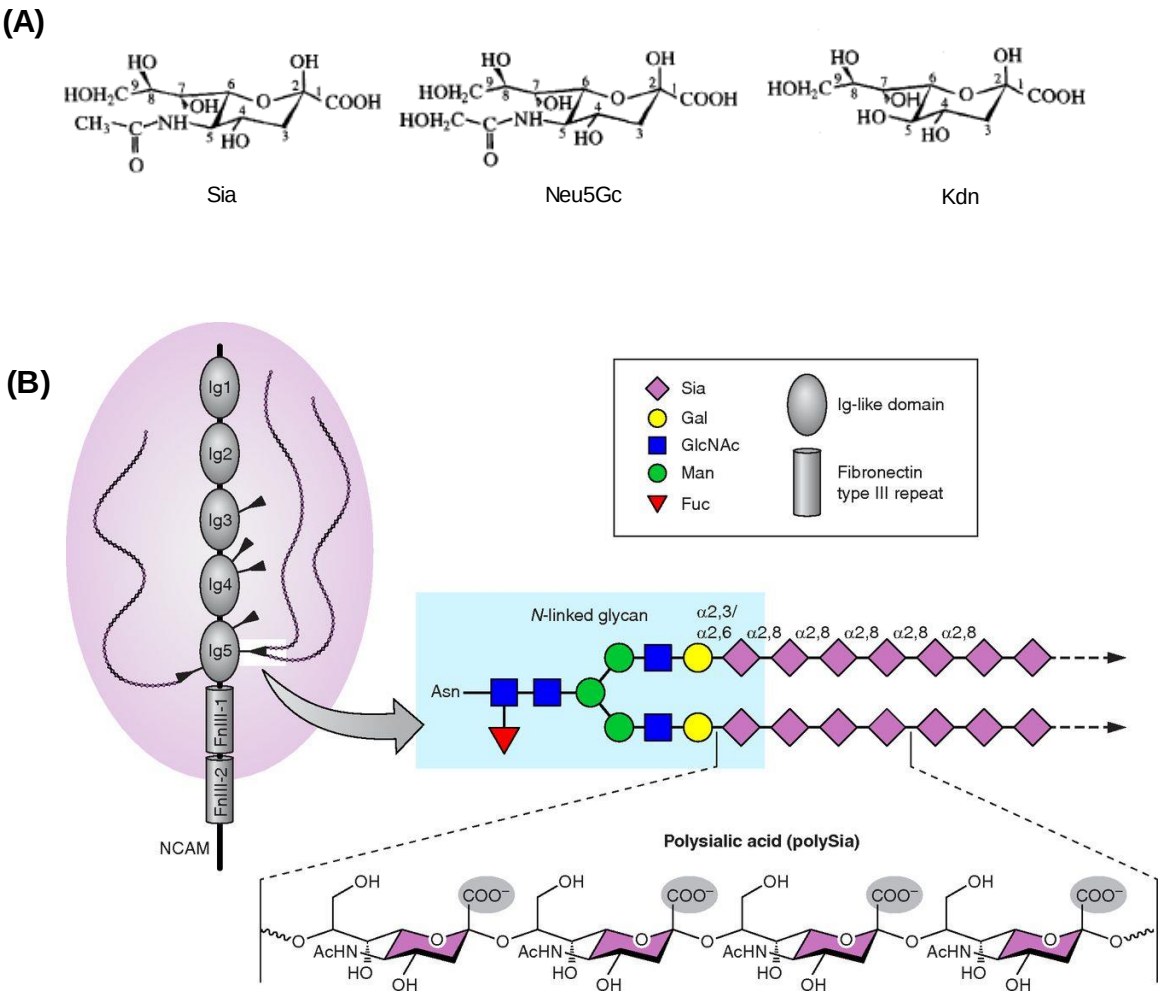


Figure 1.3: Schematic presentation of polysialylated NCAM.

(A) Polysialic acid three main building blocks, including 5-*N*-acetylneuraminic acid (Sia), 5-*N*-glycolylneuraminic acid (Neu5Gc) and 5-deamino-3,5-dideoxyneuraminic acid (2-keto-3-deoxynonulosonic acid, Kdn). Taken from (Mühlenhoff et al., 1998). (B) Representation of the polysialylated neural cell adhesion molecule (NCAM). NCAM has six *N*-glycosylation sites (black arrows) and 5th and 6th *N*-glycans carry one or more polySia chains, a linear homopolymer of α-2,8-linked sialic acid. PolySia chains are linked with α-2,3 or α-2,6 first and have more than 90 monomers forming polyanions (grey spheres highlighted negatively charged carboxyl group). This increases the hydrodynamic volumes of NCAM and effect the carrier binding such as polySia binding to NCAM changes the adhesion to repelling molecule. From (Schnaar et al., 2014).

1.6.2 PolySia biosynthesis by polysialyltransferases

The biosynthesis pathway for polySia begins in the cytosol, where UDP-*N*-acetylglucosamine (UDP-GlcNAc) is converted to *N*-acetylmannosamine (ManNAc). In mammals, ManNAc is phosphorylated forming ManNAc-6-phosphate (ManNAc-6P). The condensation reaction of ManNAc or ManNAc-6P with phosphoenolpyruvate (PEP) forms neuraminic acid or neuraminic acid-9P. Then, neuraminic acid-9P is dephosphorylated resulting in neuraminic acid (Sia). The final reaction occurs in the nucleus, involving the conjugates of Sia to cytidine monophosphate (CMP-Sia) and cytosine triphosphate (CTP).

CMP-Sia enters the Golgi lumen using the CMP-Sia transporter (CMP-SiaTr), acting as a substrate donor to more than 20 human sialyltransferases (STs) that incorporate into the non-reducing end of glycans (Ivan Martinez-Duncker, 2011).

Polysialyltransferases (polySTs) use the activated Sia as a substrate to form polysialylated NCAM inside the Golgi apparatus (Bork et al., 2005) and added to glycans via α-2,8 linkages. PolyST belongs to the wider sialyltransferase family, which also has α-2,3-ST and α-2,6-ST. For that reason, polySTs are responsible for the synthesis of polySia by adding α-2,8-linked Sia to an α-2,3-linked Sia followed by multiple α-2,8-linked Sia residues (Angata et al., 1998).

PolySia is synthesised by two polysialyltransferase (polyST) enzymes, named PST (ST8Sia IV) and STX (ST8Sia II) respectively reviewed in (Tsuji, 1996, Angata and Fukuda, 2003, Mühlenhoff et al., 1998). PST and STX appear to

share enzymatic properties and have 59% sequence identity at the amino acid level. Each contains features typical of sialyltransferases including the stem domain, catalytic domain and transmembrane domains shown in **Figure 1.4** (Gascon et al., 2007). They also have polySTs motifs named polysialyltransferase domain (PSTD) and the polybasic region (PBR). The PSTD activity depends on 32 amino acids motifs, which is required to form polysialylation capacity shown in **Figure 1.4B** (Nakata et al., 2006).

The expression of enzymes is tissue-specific and cell-specific, suggesting distinct roles during development (Angata and Fukuda, 2003, Ong et al., 1998). PolySia is added by both polyST enzymes on the Ig5 domain of NCAM, which contains six *N*-linked glycan sites (**Figure 1.4A**) (Angata et al., 1998).

Angata et al (1998) attempted to determine the individual roles of the polyST enzymes and revealed that both enzymes synthesised polySia efficiently on NCAM. Their results showed the polyST enzymes specificity on *N*-glycan structures in NCAM.

Interestingly, polySTs also have auto-polysialylation activity and it has been hypothesised that polySia chains are transferred *en bloc* to the acceptor (NCAM) but the auto-polysialylation role is still indistinct (Angata and Fukuda, 2003). Mutagenesis studies carried out on auto-polysialylation showed a significant reduction of polySia-NCAM biosynthesis, suggesting the link between the synthesis of polySia on NCAM and auto-polysialylation (Mühlenhoff et al., 1998, Close et al., 2001).

Angata and Fukuda (2003) later proposed that PST and STX work cooperatively and their mutual presence results in higher and longer polySia polymer chains. Both PST and STX produce polySia but the degree of polysialylation for STX (~40 sialic acid residues) was lower than that observed with PST (~60 sialic acid residues) (Angata and Fukuda, 2003, Gascon et al., 2007).

STX has been proposed as a potential marker to detect metastatic neuroblastoma cells and is thought to be the major enzyme that synthesises polySia in tumours (Cheung et al., 2006).

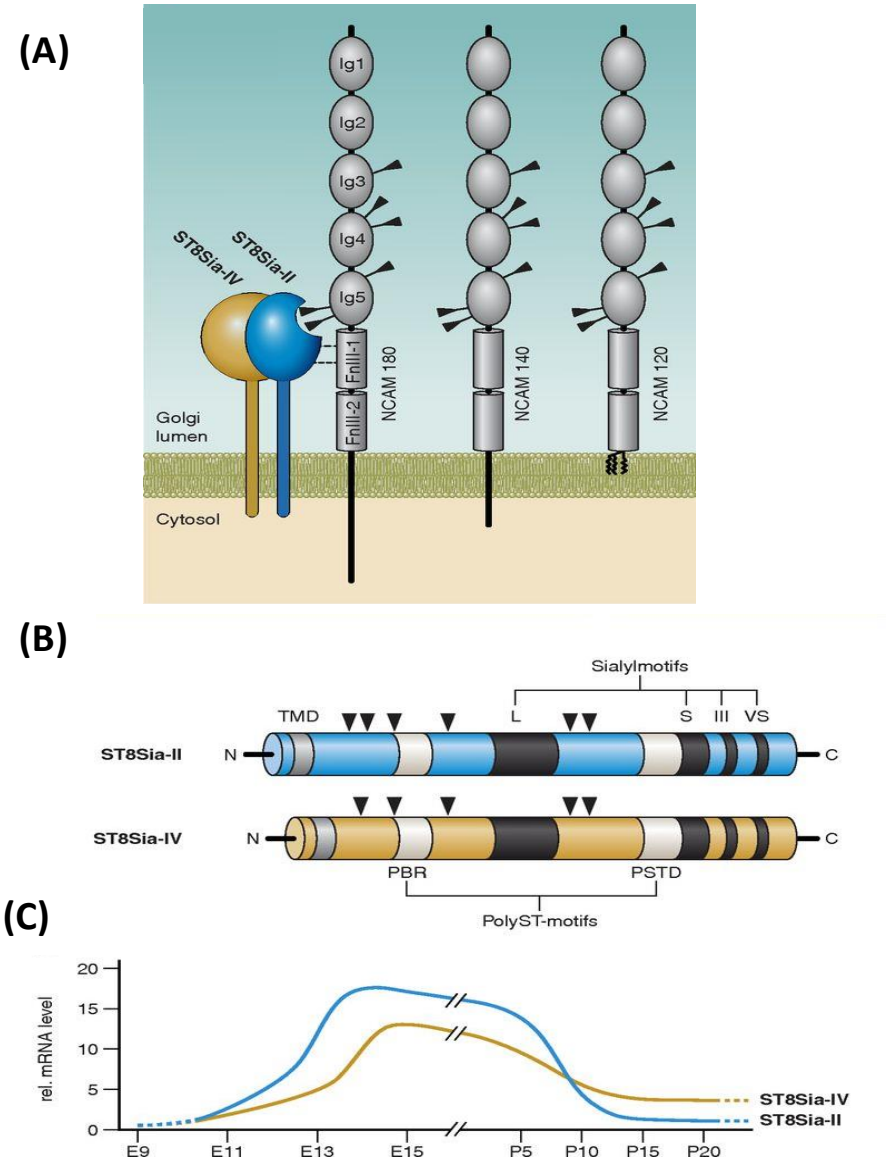


Figure 1.4: Presentation of polysialyltransferase enzymes.

(A) NCAM is the major polySia binding acceptor. *In vitro*, all isoforms are polysialylated on the Ig5 domain. (B) Schematic presentation of polysialyltransferases STX and PST. Both enzymes consist of a stem region, a transmembrane domain (TMD), an NH_2-terminal cytosolic domain and a COOH-terminal catalytic domain. Black triangles represent *N*-glycosylation sites. Black boxes indicate the sialyl motifs: large (L), short (S), motif III (III), and very short (VS) that are conserved in all mammalian sialyltransferases). (C) The overview of the polyST enzyme mRNA level during mouse brain development studied by quantitative real-time RT-PCR using whole mRNA extracts from the brain. Adapted from (Schnaar et al., 2014)

1.6.3 Polysialylated NCAM

The most common modification of membrane secreted proteins and glycan residues is glycosylation, which plays an important role in protein folding, stability and function (Varki, 1993). This is true for NCAM, which is particularly subject to post-modification by polySia, expressed predominately during the embryonic development stage. NCAM glycosylation occurs in the endoplasmic reticulum (ER) and Golgi apparatus (for details, see review (Kiss and Rougon, 1997). Polysialylated-NCAM (polySia-NCAM) plays an important role in neural development and plasticity (Rutishauser and Landmesser, 1996, Kiss and Rougon, 1997, Jensen and Berthold, 2007).

In organogenesis, polySia is expressed in mesodermal and endodermal derivatives; NCAM doesn't carry polySia at this time. On the embryonic (E) day 8-8.5 in mice, NCAM is polysialylated later in the prenatal phase until E9 when all NCAM is polysialylated, reviewed in (Hildebrandt et al., 2010, Bonfanti, 2006, Brocco et al., 2003). Within week 1, the expression of polySia decreases, NCAM isoforms NCAM-140 and NCAM-180 appear without polySia whereas NCAM-120 appears during the postnatal regulation of the brain (Hildebrandt et al., 2010).

In the developing brain, NCAM is highly polysialylated and found abundantly (Angata and Fukuda, 2003). This results in NCAM having various roles in promoting cell adhesion, cell migration, neurite outgrowth, branching, neuronal path finding and synapse formation (Bonfanti, 2006, Brusés and Rutishauser,

2001, Gascon et al., 2007, Kiss and Rougon, 1997, Rutishauser and Landmesser, 1996).

Polysialylated NCAM is also constantly present, albeit at significantly lower levels in restricted areas of the brain, which are the olfactory bulb and hippocampus, where the neural generation and plasticity persist (Angata and Fukuda, 2003, Rutishauser and Landmesser, 1996). PolySia's role in cell-cell interaction has been revealed using the NCAM deficient mice and endo-N treatment, which covered the requirement for polySia for olfactory neuroblast migration (Angata and Fukuda, 2003, Cremer et al., 1994). These indicate the role of NCAMs in the regeneration and the development of neural cells.

1.6.4 PolySia functional roles and its cellular interactions

Various *in vitro* and *in vivo* studies (knockdown mice of polySia/NCAM) give evidence of several roles of polySia in cell interactions reviewed in (Gascon et al., 2007, Hildebrandt et al., 2010, Schnaar et al., 2014, Rutishauser, 2008, Hildebrandt et al., 2007). PolySia has a negative charge and the attachment of polySia on NCAM increases the hydrodynamic radius, which causes an increase in the intermembrane space and disrupts the adhesion properties of NCAM (**Figure 1.5A**) (Brusés and Rutishauser, 2001, Fujimoto et al., 2001, Johnson et al., 2005). In the case of polySia removal, using endo-N results firm binding between two NCAM molecules (Hoffman and Edelman, 1983, Sadoul et al., 1983).

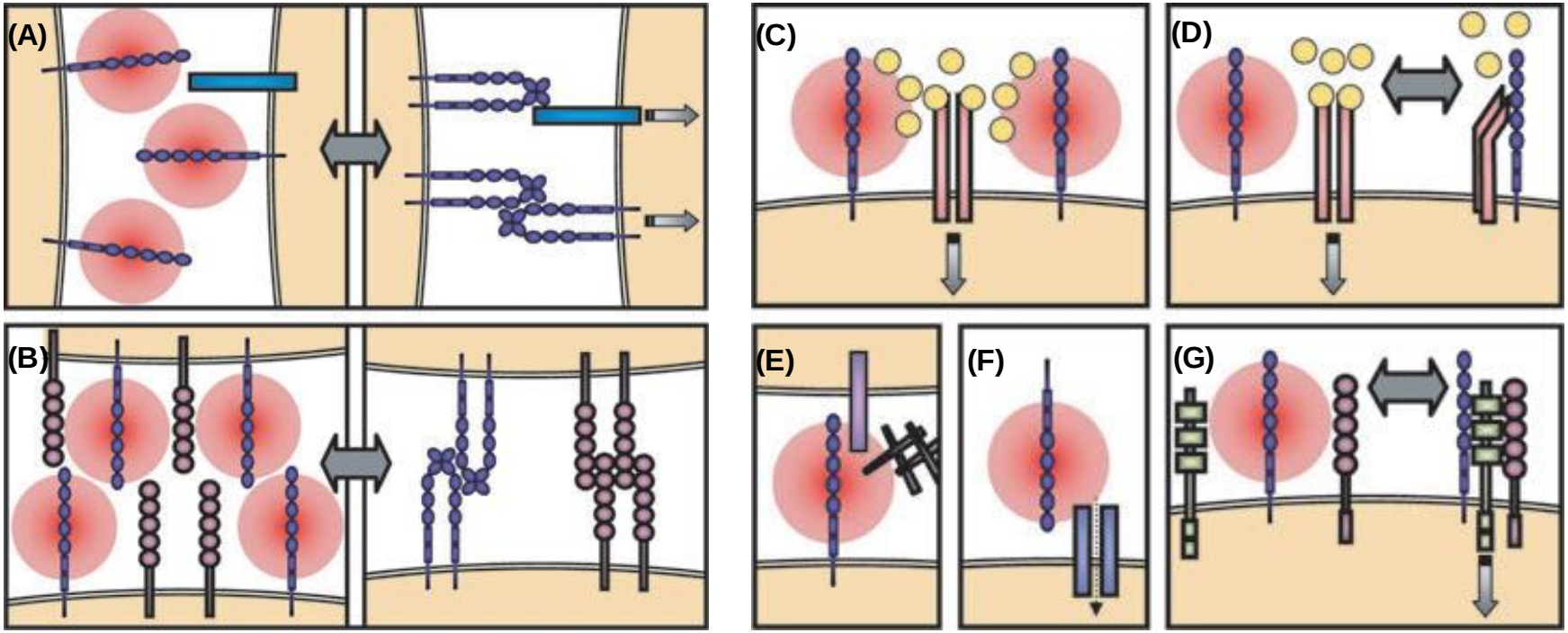


Figure 1.5: Different modes of polySia cell interactions.

(A) PolySia shields neural cell adhesion molecule (NCAM) and removal of polySia induces NCAM homo- or hetero-signalling. (B) PolySia acts as a negative regulator preventing NCAM interaction with other molecules (integrin). (C) PolySia initiates the interaction of cell surface receptors (brain-derived neurotrophic factor with TrkB) by acting as a scavenger molecule. (D) PolySia also inhibits NCAM signalling or (E) polySia binding to other cell surface or cell matrix molecules (glycosaminoglycans). (F) PolySia modules activation of ionotropic glutamate receptors or formation of NCAM signalling complexes formation (G). Modified from (Hildebrandt et al., 2007).

The presence of polySia on NCAM regulated NCAM-mediated adhesion, which prevents cell contacts, suggesting the impact of polySia on neurite outgrowth. Study of polySia negative mouse models has revealed that the important role of polySia is to shield NCAM and is associated with the organisation, timing and site-specificity of NCAM interactions (Hildebrandt et al., 2007). The role of polySia in NCAM adhesion has been proposed, which is that it causes a steric inhibition of cell to cell apposition ((Rutishauser and Landmesser, 1996); **Figure1.5B**). This model suggests that the charge and the hydration nature of polySia hinder NCAM interactions either with NCAM or with other molecules including integrins (Fujimoto et al., 2001, Johnson et al., 2005, Yang et al., 1994). The removal of polySia allows cell interaction and has been widely studied using polySia and NCAM mouse models and also the impact of polySia on fasciculation, branching and axons (Rutishauser and Landmesser, 1996, Weinhold et al., 2005).

Besides this, there is considerable evidence for the role of polySia in the developing nervous system. The presence of polySia on the neurons of the hippocampus and hypothalamus becomes more sensitive to brain-derived neurotropic factors, which gives the indirect explanation of the role of polySia as a scavenger molecule (**Figure 1.5C**) or polySia inhibiting the NCAM *cis*-interaction (**Figure 1.5D**) (Hildebrandt et al., 2007). Therefore, loss of polySia promotes NCAM formation (**Figure 1.5 A, B**) or modulates NCAM *cis*-heterophilic interactions, however further experimental validation for these proposed models is required (Kiselyov et al., 2005).

NCAM is not only an adhesion molecule but also plays a role in intracellular signalling. NCAM functions in a Ca^{2+}-independent manner and mediates cell-cell (homophilic (NCAM-NCAM), heterophilic (NCAM-fibroblast growth factor receptor (FGFR)) and cell-matrix interactions in the cell (including neural) (Gascon et al., 2007, Kiryushko et al., 2006). The NCAM crystal structure reveals the involvement of Ig1 and Ig2 in *cis* interactions (same cells) whereas Ig3 mediates the NCAM *trans* interactions (opposing cells), which instantaneously binds to Ig1 and Ig2 forming a zipper like NCAM adhesion complex (Soroka et al., 2003). These interactions activate the intercellular responses which are cell differentiation, growth, survival and many other cellular mechanisms (Soroka et al., 2003).

Nearly twelve years ago, the functions of NCAM with FGFR was suggested to induce neurite outgrowth (Kiselyov et al., 2005). Several studies suggested polysialylation of NCAM stimulates FGFR dimerization upon binding. This activates several proteins resulting in various downstream signalling pathways (Walsh and Doherty, 1997). Mouse models have shown the role of polySia affecting NCAM interactions whereas neuroblastoma cells without polySia promote NCAM trans-interaction at cell contact sites by reducing proliferation and result in the activation of the extracellular signal-regulated kinase (ERK) pathway (Seidenfaden et al., 2003).

Studies of neuroblastoma cells and the subventricular zone (SVZ) derived interneuron precursors which demonstrated the role of polySia-regulated and ERK1/2-dependent heterophilic signalling results in neuronal differentiation and

survival of neuroblastoma cells (Seidenfaden et al., 2003, Seidenfaden et al., 2006). Similar findings were found in *in vivo* studies with polySia removal by endo-N stimulating neuronal differentiation of SVZ derived precursors in explant cultures and *in situ* (Petridis et al., 2009).

In addition, loss of polySia reduced cell motility and enhanced focal adhesion of tumour cells. In both cases, effects occurred in an NCAM-dependent manner (Eggers et al., 2011).

NCAM homophilic interactions also stimulate neurite extension (Rønn et al., 1998) and play an important role during development through changes in NCAM expression (Rutishauser and Landmesser, 1996). Several studies indicate the role of NCAM in learning and memory, especially the post-translationally modified NCAM by polySia in the adult brain, which have been shown to be associated with learning (Rønn et al., 1998).

Cancer cell function is affected by NCAM signalling, for example, transfection of rat glioma cell line (BT4Cn) with human NCAM-140 significantly reduced tumour invasiveness *in vivo* and the expression of NCAM in pancreatic β-cell tumour abolished the metastasis formation (Edvardsen et al., 1994, Perl et al., 1999).

1.6.5 Clinical expression of polySia in cancer

Different expression of sialylated phenotypes has been employed to differentiate between benign and malignant tumours (Ivan Martinez-Duncker, 2011). Studies have suggested a role for polySia whereby polySia-NCAM modulates tumour cell adhesion and facilitates cell detachment, the first stage of the metastatic cascade.

The expression of polySia has been shown to correlate with tumour progression, metastasis and invasion in metastasis-prone malignant tumours, including neuroblastoma, glioma, rhabdomyosarcoma, small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC), as summarised in **Table 1.3.**

Table 1.3: *In vitro* evidence of the role of polysialylated NCAM in tumours.

Tumour name	No. of patients	PolySia expression (%)	PolySia-NCAM relation with tumour	Reference
Neuroblastoma	24	63	Diagnosis and prognosis marker	(Glüer et al., 1998b)
	136	100	STX mRNA clinical utility as a molecular marker	(Cheung et al., 2006)
	5	60	Marker for differential diagnosis	(Glüer et al., 1998a)
	36	58	PolySia-NCAM is a significant molecular marker	(Korja et al., 2009)
Astrocytoma	30	100	Highly malignant astrocytoma express more polySia	(Petridis et al., 2009)
Rhabdomyosarcoma	11	100	PolySia-NCAM as a diagnostic and prognostic marker	(Gluer et al., 1998c)
Wilm's	25	100	Onco-developmental antigen	(Roth et al., 1988b)
	9	100	Onco-developmental antigen	(Roth et al., 1988c)
	5	100	PolySia-NCAM is an onco-developmental antigen in kidney	(Roth et al., 1988a)
	2	100	useful marker for differential diagnosis	(Glüer et al., 1998a)
Small cell lung cancer (SCLC)	17	60	NCAM with polySia as a prognostic factor	(Miyahara et al., 2001)
	70	93	NCAM-polySia expression could favour metastatic spread	(Lantuejoul et al., 1998)
	25	100	PolySia can be used to distinguish two neuroendocrine lung tumours (i.e. SCLC and carcinoids)	(Komminoth et al., 1991)
	50	100	High polySia expression correlates with reduced cell-cell adherence and metastasis	(Scheidegger et al., 1994)
Non-small cell lung cancer (NSCLC)*	57	49	PolySia and STX as a clinical marker	(Tanaka et al., 2000)
	236	19	clinical marker	(Tanaka et al., 2001)
Other lung cancer	50	72	Poor differentiation and aggressive clinical behaviour	(Lantuejoul et al., 1998)

Tumour name	No. of patients	PolySia expression (%)	PolySia-NCAM relation with tumour	Reference
Glioma	30	30	PolySia facilitates tumour invasion of glioma in the brain	(Suzuki et al., 2005)
Glioblastoma (GBM)	56	70	PolySia-NCAM represents a valuable biomarker for the prognosis of GBM patients	(Amoureux et al., 2010)
Medulloblastoma	29	100	PolySia-NCAM a new biologic marker	(Figarella-Branger et al., 1996)
Nephroma	8	38	Renal onco-developmental marker	(Nadasdy et al., 1993)
Medullary thyroid	33	100	PolySia of NCAM is a valuable marker to distinguish medullary carcinomas from other types of thyroid carcinomas	(Komminoth et al., 1994)
Pancreatic	15	67	Expression correlates with tumour proliferation and invasion	(Kameda et al., 1999)
Multiple myeloma	70	80	Valuable marker for monitoring therapy	(Kaiser et al., 1994)
	92	61	Tumour prognostic marker	(Smith et al., 1996)
Colorectal cancer	39	87	Importance of polysialylated NCAM in tumour development	(Fernández-Briera et al., 2010)
Pituitary tumours	82	46	Polysialylated NCAM is strongly related to tumour invasion	(Trouillas et al., 2003)
	12	36	Prognostic factor for pituitary tumours	(Wierinckx et al., 2007)
**(carcinoids, atypical carcinoids, and neuroendocrine carcinomas)*				

Many studies have focused on trying to understand the role of the polysialylated NCAM in neuroblastoma (Glüer et al., 1998a, Glüer et al., 1998b, Hildebrandt et al., 1998). Neuroblastoma is highly malignant and in metastatic childhood cancer it has been reported that most neuroblastoma express on NCAM-180 (Winter et al., 2008). Therefore, polySia-NCAM is considered as an onco-developmental antigen which is expressed not only in other malignant neuro-dermal and neuroendocrine tumours but also in neuroblastoma (Hildebrandt et al., 1998).

Polysialylated NCAM expression was studied in neuroblastoma patients showing high level of polySia-NCAM in advanced clinical stages of the disease and this result was correlated with other neuroblastoma prognostic factors including *MYCN* gene amplification and serum concentrations (Glüer et al., 1998b). In addition to this, the serum levels correlated with the polysialylated NCAM contents of the neuroblastoma and thus is a useful prognostic and diagnostic neuroblastoma marker (Gluer et al., 1998d).

Furthermore, neuroblastoma cells treated with endo-N showed a significant reduction in cell migration, indicating the impact of polySia removal on the progenitor cell biology (Petridis et al., 2009). Studies have illustrated the presence of polySia promoting migration in neuroblastoma and rhabdomyosarcoma cells, providing evidence for the initiation of NCAM interaction when polySia is removed and this was consistent with the *in vitro* studies (Eggers et al., 2011).

It has previously been mentioned that polySia synthesis is carried out by PST and STX whereas STX mRNA expression has been reported to be a potential molecular marker of metastatic neuroblastoma (Cheung et al., 2006). STX has been described as a novel molecular marker to detect tumours and is thought to be a key regulator of synthesis polySia in tumours. In particular, transfection of C6 glioma cells with STX in rats showed an increase in polySia expression promoting invasion to the corpus callosum when inoculated into the brain, which was rarely seen in mock-transfected C6 cells describing the close correlation of polySia and tumour invasion (Suzuki et al., 2005).

PolySia re-expressed in malignant tumours e.g. Wilm's tumour, has been reported first and studied using anti-monoclonal polySia antibody (mAb 735), which described the presence of polySia on NCAM as an 'onco-developmental antigen' (Fernández-Briera et al., 2010, Roth et al., 1988c).

Daniel et al (2001) injected endo-N into mice with tumours strongly expressing polySia-NCAM, this showed a decrease in polySia expression and established the relationship between polySia expressed in the tumour cells and metastasis. Similar work was studied on different subsets of cells with different phenotypes found that polySia positive tumour cells produced more intracutaneous metastasis that low expressing polySia cells when nude mice was injected subcutaneously (Scheidegger et al., 1994).

Polysialylated NCAM is considered a prognostic factor in lung cancers (both small cell and non-small cell) where Trouillas et al (2003) analysed the

polysialylated NCAM expression in 82 pituitary tumours and found polysialylated NCAM in 42.3% of typical pituitary tumours and also these tumours were endoN sensitive and suggested a strong correlation with tumour invasion. Furthermore, two studies carried out with non-small cell lung cancers demonstrated the clinical significance of polySia, especially the presence of the polyST enzyme STX, which is associated with the clinical development and could be used potentially either as a clinical marker or new cancer therapeutic target (Tanaka et al., 2001, Tanaka et al., 2000).

PolySia expression is also seen in other neuroendocrine lung tumours where polySia expression is associated with higher stages of disease. 17 surgically SCLC specimens were studied using immunohistochemistry and showed 60% polysialylated NCAM expression, whereas other studies showed 93% polySia expression in 70 SCLC tumours suggesting the NCAM-polySia is regarded as a prognostic factor in SCLC tumours (Lantuejoul et al., 1998, Miyahara et al., 2001). Altogether, clinical expression of polysialylated NCAM in various tumours functions as an onco-developmental antigen. This expression of polySia contributes to tumour cell detachment, tumour growth, invasion and finally to metastasis.

1.6.6 STX inhibition by biochemically engineered sialic acids

PolySia is ectopically expressed in tumours and is thought to be playing a role in tumour invasion as described in previous sections. The expression of STX is thought to be responsible for NCAM polysialylation in tumours. Hence, inhibiting polySia synthesis can be an anti-tumour agent against these tumours. One approach demonstrated by Bertozzi's group that treating cells with sialic acid precursor molecules i.e. *N*-butylmannosamine. This precursor inhibited the polysialyltransferases activity and can be applied to polySia expressing tumours especially neuroblastoma can be potentially treated with the modulation of polysialylated NCAM by altering polySia expression (Mahal et al., 2001). Also, novel sialic acid precursors, *N*-propanoyl mannosamine (ManNProp) cause selective inhibition of STX driven by polysialylated NCAM (Horstkorte et al., 2004a).

Recently, investigations have been carried out using the epilepsy therapy drug known as Valproic acid (VPA, 2-propylpentanoic acid), which showed a link with anti-cancer activity. It is shown that VPA treatment results in metastatic down-regulations of tumour cells in different experimental conditions and depends on NCAM modulation (Bork et al., 2007). Work done by Beecken et al (2005) showed RT-PCR results that VPA up-regulates PST and down-regulates STX at mRNA levels. In this study, small molecule polyST inhibitors have been employed and their effect on polySia has been studied, in reflection to cell functions (particularly invasion) and more details are given are chapter 4.

1.7 Aims and objectives

Tumour dissemination (adhesion, migration and invasion) is the main cause of the failure of cancer therapy resulting in high mortality. Thus, there is much interest in developing therapeutic strategies which can control tumour dissemination.

As has been reviewed here, expression of polySia on NCAM reduces the adhesiveness of tumour cells and promotes tumour dissemination. This is strongly related with poor clinical prognosis and correlates with tumour aggressiveness and invasion in neuroblastoma and several other tumours.

Thus it is hypothesised that by targeting the expression of polySia (i.e. by removal/reduction) it should allow control of tumour dissemination. One way of doing this is by inhibiting polySia synthesis in the first place by developing inhibitors of the enzyme. This will in turn reduce cell-surface expression of polySia found on cancer cells, increasing cell-cell and cell-matrix adhesion, and thus reduce the opportunity for tumour cell dissemination. This strategy has been adopted at the Institute of Cancer Therapeutics (ICT), where small molecule inhibitors of polyST are being designed and synthesised.

In this project, the aims are to establish specific *in vitro* tests to evaluate these new molecules in a panel of characterised cell lines.

These aims will be achieved by addressing the following objectives:

- Assembly and characterisation of a panel of tumour cell lines having a range of polySia and polyST expression, in terms of growth parameters and expression of polySia and other sialic acids.

- Development and validation of *in vitro* assays to assess the effects of polyST inhibition on polySia decoration of NCAM and on cell invasion in both 2D and 3D models.

- Screening and evaluation of the potential effect of novel inhibitors of polyST developed 'in-house' on the inhibition of polySia expression and tumour cell invasion.

CHAPTER TWO

2 Assembly and characterisation of a cell line panel for screening polySTi

2.1 Introduction

As reviewed in Chapter 1, in high-risk neuroblastoma patients, the overall survival rate is poor. Despite new advances in treatment, relapse is the biggest obstacle to successful therapy. As previously described, the expression of polysialylated NCAM correlates with neuroblastoma prognostic and diagnostic markers (**Table 1.3**). Therefore, the strategy proposed here is that by developing synthetic inhibitors of polyST (STX), this will reduce neuroblastoma progression and metastasis. One of the major aims of neuroblastoma treatment is to develop therapies targeting the cancerous cells. For that reason, the

selection of suitable relevant and validated models is highly important for the evaluation of novel drugs.

The screening panel should include cells expressing the molecule(s) of interest, ideally at different levels i.e. high, medium, low or null. In this case, the molecules of interest are polySia and two polyST enzymes (PST and STX). For this study, five cancer cell lines were selected for investigation based on previous studies both from the literature and in-house. There are no specific antibodies available that can detect polyST. However, previous studies have shown a direct association between polySia with polyST expression (Hildebrandt et al., 1998). Therefore, the effect of inhibition of the enzyme can be detected by monitoring polySia expression. For this investigation, a mixture of naturally expressing cell lines and isogenic lines were selected for the screening of polySia expression.

A pair of isogenic rat glioma cells were utilised here where the only difference is that one has been engineered to express polyST enzyme (i.e. STX) (Suzuki et al., 2005) and as a consequence result in polySia expression. Isogenic cells display high genetic similarity with a unique feature (Bajaj et al., 2009). They are useful as they are able to distinguish and exploit the difference between polySia expression shown in house and in the literature.

As neuroblastoma is the cancer of interest, two human neuroblastoma cell lines IMR-32 and SH-SY5Y have been selected, which are polyST, polySia and NCAM positive; this is based on earlier studies (Valentiner et al., 2011, Al-Saraireh et al., 2013). Previously, high levels of polySia and STX expression in

C6-STX and IMR-32 were shown compared to SH-SY5Y, whereas PST levels were barely detected in IMR-32 cells only (Al-Saraireh et al., 2013).

Finally, the colorectal adenocarcinoma cell line DLD-1, was studied as a negative control. These cells do not express polyST, polySia or NCAM as revealed previously (Jimbo et al., 2001, Al-Saraireh et al., 2013).

Various tools for studying polySia expression have been described in the literature. Functional methods to study polySia related to this study include, endoneuraminidase N (endo-N), anti-polySia antibodies and endo-N-GFP, and their interactions with polySia is shown in **Figure 2.1**.

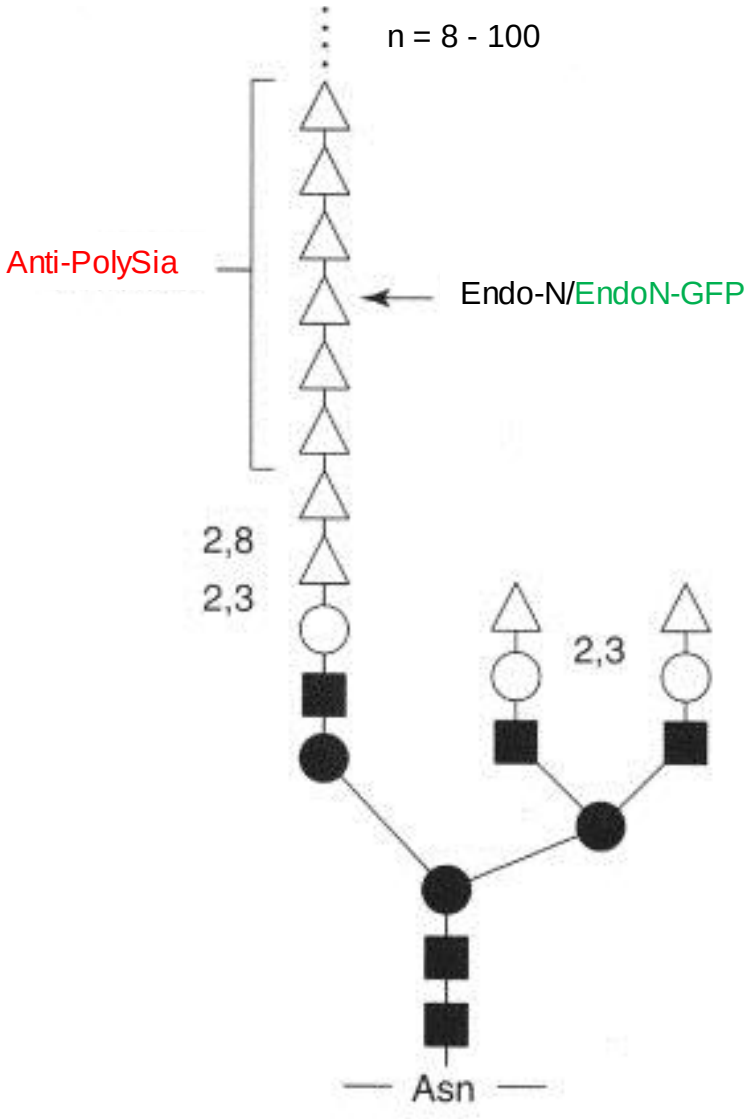


Figure 2.1: Tools for the study of polySia.

PolySia is detected by anti-polySia antibody, endo-N and endoN-GFP. (Endo-N: Endoneuraminidase; GFP: Green Fluorescent Protein). Modified from (Rutishauser and Landmesser, 1996).

Endo-N has been used as a biological probe for specific and rapid degrading of α-2,8-glycosidically linked polymers of sialic acid (i.e. polySia) with a chain length not less than seven to nine residues (Finne and Mäkelä, 1985, Rutishauser et al., 1985). This enzyme has been shown to recognise and specifically remove polySia from the rat neonatal neural membrane (Vimr et al., 1984). Endo-N treatment has been used to reduce expression of polySia *in vitro* and *in vivo* in order to understand the structural and functional properties of polySia in the regulatory process of cell migration, cell adhesion, neurite outgrowth, branching, axon path-finding and synapse formation (Angata and Fukuda, 2003). For that reason, endo-N is a useful tool to study direct or indirect degradation of polySia in various conditions and assays including removal of polySia from living cells and analysing the recovery of polySia re-expression (Chapter 3; (Al-Saraireh et al., 2013)).

Relatively few anti-polySia antibodies have been reported for the detection of polySia as shown in **Table 2.1**. First is the H.46 antibody used for the detection of polySia, following endo-N treatment (Vimr et al., 1984). Other commonly used antibody is mAb 735 raised in NZB nude mice against B meningococcal antigen and binds to polySia with eight or more residues (Frosch et al., 1985). Earlier studies have shown the use of H.46 and mAb 735 for polysialylated NCAM characterisation (Frosch et al., 1985, Livingston et al., 1988, Vimr et al., 1984). Other mouse monoclonal antibodies are mAb OL.28, mAb 2-4B and mAb 2-2B, which have been used for the identification of polySia in various cancer cell lines are listed in **Table 2.1**.

In this thesis, anti-polySia antibody (mAb 735) has been used. The specificity of mAb 735 has been extensively characterised in previous studies (Frosch et al., 1985). MAb 735 does not strictly favour the recognition of polySia chains forming extended helices but has a high affinity to recognise longer polySia chains i.e. every three sialic acids units in a paired manner (Husmann et al., 1990, Nagae et al., 2013). Therefore, it considered a specific probe for the definite polySia detection.

Another polySia recognising probe was developed in which bacteriophage-derived endo-sialidase was catalytically inactive and fused to green fluorescent protein (GFP). This construct binds to polySia but does not possess catalytic activity (i.e. it does not cleave polySia). This construct can be used as an alternative to the anti-polySia antibodies. EndoN-GFP is a single step labelling tool which has high specificity for polySia (Jokilammi et al., 2004). Studies have shown that both endoN-GFP and mAb 735 have the same labelling pattern and both have been used to characterise neuroblastoma tissues (Jokilammi et al., 2004, Korja et al., 2009).

Table 2.1: List of anti-polySia antibodies and their features.

Antibody	Monoclonal (Mono)/ polyclonal (poly)	Type (Ig)	Source	PolySia chain recognition	Antigen species	Degree of polymerisation	Reference
H.46	Poly	IgM	Horse	Sia/Neu5Gc	Meningitidis Group B bacteria	≥8	(Allen et al., 1982) (Sato et al., 1995)
735	Mono	IgG	Mouse	Sia/Neu5Gc	Meningitidis Group B bacteria	≥8	(Frosch et al., 1985) (Sato et al., 1995)
12E3	Mono	IgM	Mouse	Sia	Embryonic rat forebrain	≥5	(Sato et al., 1995)
OL.28	Mono	IgM	Mouse	Sia	Rat oligodendrocytes	≥4	(Martersteck et al., 1996)
2-4B	Mono	IgM	Mouse	Neu5Gc	Autoimmune mice	≥2	(Sato et al., 1998)
2-2B	Mono	IgM	Mouse	Sia	Meningitidis Group B bacteria	≥3	(Rougon et al., 1986)

As described in Chapter 1, it is important to establish selectivity of polyST inhibitors. There is a chance that polyST inhibitors may lack total selectivity, thereby inhibiting α-2,3 and α-2,6-STs. This is significant since sialylation is carried out by twenty different sialyltransferases, which catalyse the different glycosidic linkages through α-2,3- or α-2,6- bonds to galactose, *N*-acetylgalactosamine or α-2,8-bonds to other molecules resulting in polySia as mentioned in section 1.6.2 (Harduin-Lepers et al., 2012).

Sialic acids play extensive roles in physiological and pathological processes, including cancer, immune response regulation and pathogen binding (Varki and Varki, 2007). For example α-2,3 sialic acid is involved in myelin formation and maintenance (Kleene and Schachner, 2004). Two recognition molecules, L1 (immunoglobulin superfamily) and CD24 (a highly glycosylated mucin type glycoprotein) work cooperatively in neurite outgrowth and signal transduction pathway, and the binding of or inhibition of neurite outgrowth (Kleene et al., 2001).

In addition, these sialic acids also play a significant role in pathological processes. For example, α-2,3 sialic acid is the main glycan involved in H5N1 'bird flu' virus and humans have resistance to this infection due to the display of α-2,6 sialylated glycans on the epithelium of the upper respiratory tract (Varki and Varki, 2007). Aberrant expression of a-2,6-ST has also been reported in many human malignancies, whereby expression of *ST6GAL1* gene is found in colon, breast and cervical cancers, acute myeloid leukaemia and some brain tumours reviewed in (Dall'Olio and Chiricolo, 2001).

There are various methods available which can be used to detect and identify various linkages by which sialic acids are joined together to generate diversity (Varki, 2007, Knibbs et al., 1991). This includes plant lectins, antibodies, recombinant soluble microbial proteins and recombinant soluble mammalian receptors as reviewed in (Varki and Varki, 2007).

Lectins have specificity for linkages (e.g. α-2,6 sialic acids are recognised specifically by SNA) and can distinguish between *N*-glycans and *O*-glycans (Cumings et al., 2009) unlike antibodies or other methods. Furthermore, tumour associated carbohydrates may express at a low or high level which cannot be determined by other techniques. For this, lectins are a useful tool to investigate the expression of sialic acids in a panel of tumour cells. For that reason, in this thesis, plant lectins are used to determine the specificity and distribution of native sialic acids, and the linkages of target cells at the cell surface. The information derived from lectins gives an indication of polySTi specificity.

The plant lectins are glycan-binding proteins and have been used as a powerful tool to study glycan structures (Sharon and Lis, 2004). The term lectin is derived from the Latin word *legere* meaning 'select' or to 'choose', and has been generalised to include all non-immune carbohydrate-specific agglutinins regardless of source or blood-type specificity (Sharon and Lis, 2004). Lectins were initially found in plants but later they were isolated from microorganisms and animals (Sharon and Lis, 2004). They are involved in recognition of various biological processes including metastasis, inflammation, host-pathogen interactions and exploring carbohydrates structures (Sharon and Lis, 2004). Several lectins have been reported which interact with sialic acids. The lectin

from elderberry bark and two isoform of *Maackia amurensis* seeds *Maackia amurensis* I lectin (MAL I) and *Maackia amurensis* II lectin (MAL II) have been used extensively in thesis to detect α-2,6 and α-2,3 sialic acids (Knibbs et al., 1991, Konami et al., 1994, Shibuya et al., 1987). These lectins have their own specific specificity and therefore provide useful information regarding the effect of polySTi on other linkages.

Before utilising these assays for screening polySTi, these sialic acid labelling tools need to be validated, and this was achieved using a known polySTi: Cytidine monophosphate (CMP). CMP has been used previously as a tool compound, entering the Golgi apparatus to compete with CMP-Sia, inhibit polyST and thus polySia synthesis. This has been predicted as a rate-limiting step by the earlier studies (Miyazaki et al., 2008). However, CMP is not likely to be a successful drug, since CMP is hydrophilic and charged at physiological pH. High concentrations of CMP (i.e. mM) are required to enable sufficient compounds to cross the hydrophobic cell membrane. CMP has been successfully used in-house and in the literature as a useful tool compound, effectively reducing tumour cell-surface polySia expression and cell migration (Al-Saraireh et al., 2013).

2.1.1 Aims & Objectives

In this chapter, a panel of cell lines which will be suitable for the study of the effects of polyST inhibitors (polySTi) will be assembled and characterised in terms of growth parameters and expression of polySia, NCAM and other sialic acids. These aims will be addressed by pursuing the following objectives:

- Characterisation of the cell growth parameters for each cell line using the MTT assay in order to optimise seeding densities for further experiments
- Characterisation of polySia expression in the panel
- Validation of the polySia labelling tools using a known polySTi CMP
- Determination of the distribution of other sialic acids (α-2,3 and α-2,6) in panel of cancer cell lines and validation with CMP using lectin labelling technique

2.2 Materials and Methods

2.2.1 Materials

2.2.1.1 Reagents and Solutions

All cell culture reagents and general chemicals were from Sigma-Aldrich (Poole, UK) unless otherwise stated.

Tissue culture dishes, flasks, and plasticware were purchased from Corning (Corning Amsterdam, Netherlands).

2.2.1.2 Probes and enzymes

Vectashield hardset fluorescent mounting medium with 4,6-Diamidino-2-phenylindole (DAPI) was purchased from Vector Laboratories Ltd (Peterborough, UK).

Endoneuraminidase-green fluorescent protein (endoN-GFP) was kindly provided by Professor Jukka Finne (University of Helsinki, Helsinki, Finland), and was stored at 4°C in the dark.

Monoclonal mouse IgG anti-polySia (mAb 735, clone 2) was kindly gifted by Professor Rita Gerardy-Schahn (Hannover Medical School, Germany). The polyclonal rabbit anti-mouse antibody conjugated with tetramethylrhodamine isomer R (TRITC) was purchased from Dako (Cambridgeshire, UK).

Polyclonal Rabbit anti-NCAM (ab5032) and Fluorescein isothiocyanate (FITC) conjugated goat anti-rabbit secondary antibodies were purchased from Millipore (UK) and Molecular Probes (UK) respectively.

All normal sera were purchased from Vector Laboratories (Peterborough, UK).

The specificity of the polySTi was determined using the sialic acid recognising lectins from elderberry bark, *Sambucus nigra* agglutinin (SNA/EBL) to detect α-2,6 sialic acid, and two from the legume tree *Maackia amurensis* leukoagglutinin (MAL/MAL I) or *Maackia amurensis* hemagglutinin (MAH/MAL II) to detect and α-2,3 sialic acid. Biotinylated MAL II (MAH), FITC-conjugated SNA and MAL I and fluorescent avidin kit were obtained from Vector Laboratories Ltd (Peterborough, UK) unless otherwise specified.

Cytidine monophosphate (CMP) was prepared using complete medium. CMP was obtained from Sigma-Aldrich (Poole, UK).

2.2.2 Cell lines

Human neuroblastoma cells (IMR-32 and SH-SY5Y) and colon cells (DLD-1) were obtained from American Type Culture Collection (ATCC, Manassas, USA). The glioma cell lines (C6-STX and C6-WT) were kindly provided by Prof. Minoru Fukuda (Sanford-Burnham Institute, USA).

2.2.3 Mammalian cell culture techniques

2.2.3.1 Cell culturing conditions

IMR-32 cells were grown and maintained in tissue culture treated dishes (100 mm x 20 mm) (Corning, Netherlands) containing Minimum Essential Medium Eagle medium with non-essential amino acids supplemented with 10% (v/v) fetal bovine serum (FBS), 2 mM L-glutamine, and 1 mM sodium pyruvate to form complete growth medium. All the other cell lines were maintained in 75 mm^2 cell culture treated flasks (T-75) (Corning, Netherlands).

The glioma cell lines C6-WT and C6-STX were grown and maintained in Alpha Minimum Essential medium (VWR, Leicestershire, UK) supplemented with 10% (v/v) FBS.

SH-SY5Y cells were grown to 1:1 mixture of Minimum Essential Medium Eagle with non-essential amino acids and Nutrient Mixture F-12 Ham supplemented with 10% FBS, 2 mM L-glutamine and 1 mM sodium pyruvate. DLD-1 cells were grown and maintained in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% FBS, 2 mM L-glutamine and 1 mM sodium pyruvate.

All cell lines were incubated at 37°C, 5% CO_2 in a humid atmosphere. On reaching 70-80% confluency, cells were passaged and medium was changed after 3-4 days. All cell lines were routinely checked for mycoplasma contamination using a Mycoplasma Detection ELISA Kit purchased from RnDSystems (Oxfordshire, UK).

2.2.3.2 Preparation of single cell suspension

When cells reached 70% confluence, cell culture medium was removed, and cells were washed twice with Hank's Balanced Salt Solution (HBSS) and harvested using 0.25% Trypsin/EDTA solution. Cells were incubated with trypsin for several minutes at 37°C until they began to detach. Once cells were fully detached, complete medium was added to neutralise the solution which was then centrifuged for 5 min at 1000 rpm.

The supernatant was removed and cell pellet was re-suspended in 10 ml of fresh complete medium. Cell were counted using Neubauer improved bright line haemocytometer (VWR, Leicestershire, UK) with an inverted phase contrast microscope (Olympus CK-2, x20 objective lens magnification). The haemocytometer and the coverslips were cleaned with 70% ethanol. The cleaned coverslip was placed over the wells of haemocytometer. A small volume of cell suspension (10 µl) was added at the edge of two chambers of haemocytometer and allowing fluid to go underneath the coverslip. Cells were counted in 5 squares of each chamber and an average was taken. Therefore, cell concentration per ml is calculated using the following equations:

$$\textbf{No. of cells (cells/ml)} = \textbf{average no of cells x dilution factor x } 10^4$$

Cell counts were adjusted to the required concentration by the addition of the medium and then transferred into a new flask or a petri dish containing fresh medium.

2.2.3.3 Growth curves using the MTT assay

Growth curves were determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, which used to detect cell viability and evaluate the metabolic activity of cells (Mosmann, 1983).

Cells were harvested from their exponential phase. Seeded densities between 100-100,000 cells/ml was added in 200 µl medium in round bottom 96-well plates and incubated overnight at 37°C, 5% CO_2 in a humid atmosphere. Briefly, MTT stock solution (5 mg/ml: Sigma-Aldrich) was prepared by dissolving 100 mg of MTT in 20 ml of Ultrapure water, passing through a 0.2 µm filter and storing at 4°C in the dark. MTT working solution (1:10 dilution) was prepared in complete medium and 200 µl of the MTT solution was added to the wells, each plate containing blank wells (without cells) and sample wells (with cells). Following a 4 h incubation time with the MTT at 37°C, 5% CO_2, the supernatant removed and 150 µl of dimethyl sulphoxide (DMSO) added, to dissolve the formazan crystals. The absorbance was read at 540 nm using Thermo Labsystems Multiskan Plus Microplate reader (Thermo Labsystems, UK).

From the data, the mean absorbance was calculated for each cell concentration, by subtracting the mean background absorbance from the mean absorbance of sample wells. A graph of time in cultures (days) was plotted against mean absorbance to generate the growth curves. All samples were analysed in triplicate.

2.2.4 Detection of the neural cell adhesion molecule (NCAM) using immunocytochemistry

Upon 70% cells confluency, cells were trypsinized as described in section 2.2.3.2. Cell number was adjusted ($1x10^4$ to $1x10^5$ cells/ml) and seeded onto autoclaved 22x22 mm glass coverslips in a six-well plate in 1 ml medium. The plates were incubated overnight at 37°C in a 5% CO_2 atmosphere. All experimental steps were carried out at room temperature except where stated. Once cells had adhered, the coverslips were rinsed twice with HBSS and fixed with ice cold methanol at -20°C (30 min) or 4% PFA (15 min at room temperature). The fixative was removed and slides were allowed to dry. Fix cells either used directly for immunolabelling experiments or stored at with -20°C. The optimised conditions were achieved by using different fixatives and, different concentrations of primary and secondary antibodies.

NCAM expression was characterised using immunochemistry. Briefly, cells were incubated with different concentrations of anti-NCAM polyclonal antibody prepared in phosphate buffered saline (PBS) for 30 min at room temperature. Then, the coverslips were washed 3 times for 5 min using PBS. Cells were then incubated with FITC-conjugated goat anti-rabbit secondary antibody prepared in PBS and was incubated for 30 min in the dark at room temperature. Cells were washed for 3 washes for min. Then, coverslips were mounted with Vectashield hardset fluorescent mounting medium containing DAPI as a nuclear counterstain and were kept at 4°C until further analysis.

2.2.5 Detection of polySia in cells

In this study, two polySia labelling methods were used to detect polySia expression: anti-polySia antibody (mAb 735) and endoN-GFP.

2.2.5.1 Polysialic acid detection using anti-polySia antibody (mAb 735)

An indirect immunofluorescent method was used to assess the expression of polySia in cells. When cells reached 70% confluence, cells were washed with HBSS and trypsinized as described in section 2.2.3.2. Cells were seeded onto autoclaved 22x22 mm glass coverslips in a six-well plate in 1 ml medium. The plates were incubated overnight at 37°C in a 5% CO_2 atmosphere. All experimental steps were carried out at room temperature except where stated.

Once cells had adhered, the coverslips were rinsed twice with HBSS and fixed with ice cold methanol at (-20°C, 30 min) or 4% PFA (room temperature, 15 min). Then, coverslips were air-dried and they were either stored at -20°C until use or immediately hydrated with 3 washes of PBS, pH 7.4, for 5 min each. Cells were incubated for 10 min with 1.5% normal serum prepared in PBS of the species in which the secondary antibody was raised, which here was normal rabbit serum. This blocked the non-specific binding of antibodies. Serum was removed and cells were labelled with primary mouse anti-polySia monoclonal antibody prepared in 1.5% NRS for 30 min. The coverslip was washed three times, 5 min each with PBS. TRITC-conjugated polyclonal rabbit anti-mouse antibody was added and cells were incubated for 30 min in the dark. Unbound secondary antibody was removed by washing cells three times with PBS for 5

min. Finally, coverslip was mounted with Vectashield hardset fluorescent mounting medium with DAPI onto a microscope slide and slides were stored at 4°C.

2.2.5.2 Detection of polySia expression using endoN-GFP

Polysialic acid can also be detected using a live cell antibody mimic probe, endoneuraminidase (Endo-N) tagged with green fluorescent protein (GFP), which is a catalytically inactive form of endo-N. The methodology used was adapted from (Jokilammi et al., 2004).

When cells reached 70% confluence, cells were washed with HBSS and trypsinized as described in section 2.2.3.2. Cells were seeded onto autoclaved 22x22 mm glass coverslips in a six-well plate in 1 ml medium. The plates were incubated overnight at 37°C in a 5% CO_2 atmosphere. All experimental steps were carried out at room temperature except where stated. Once cells had adhered, the coverslips were rinsed twice with HBSS and fixed with ice cold methanol at (-20°C, 30 min) or 4% PFA (room temperature, 15 min). Then, coverslips were air-dried and they were either stored at -20°C until use or immediately hydrated with 3 washes of PBS, pH 7.4, for 5 min each. The non-specific binding of cells was blocked by 1.5% Normal Horse Serum (NHS) in PBS for 1 h at room temperature. Then, different endoN-GFP concentrations were prepared in PBS was added to the cells and incubated in the dark for 1 h. Coverslips were then washed three times in PBS for 5 min and were mounted with Vectashield hardset fluorescent mounting medium containing DAPI as a nuclear counterstain and were kept at 4°C.

2.2.6 Detection of the expression of other sialic acids

As covered in the introduction section, in order to confirm specificity of polySTi, it is important to know its effects on other sialic acid expression, i.e. α-2,3 and α-2,6 sialic acids. Prior to use the methodology, MAL I, SNA and MAL II optimised as follows:

2.2.6.1 Optimisation of FITC labelled MAL I and SNA

Cells were grown onto autoclaved 22x22 mm coverslips in their required medium (stated in section 2.2.3). Cells were washed with HBSS twice and labelled with a range of concentrations of lectins (range: 2, 10, 15, 20 µg/ml) for different incubation times (15 or 30 min) and at different temperatures (i.e. room temperature or 37°C). After three washes with PBS to remove unbound lectins, cells were fixed with either 4% PFA (15 min, RT) or ice cold methanol (30 min, -20°C). The negative control was treated with diluents instead of MAL I and SNA. After fixation, cells were washed three times with 1 ml of PBS and then mounted with Vectashield hardset fluorescent mounting medium with DAPI onto the microscope slides. The slides were stored at 4°C and evaluated microscopically as described in section 2.2.8.

2.2.6.2 Optimisation of biotinylated labelled MAL II

Maackia amurensis leukoagglutinin lectin II (MAL II) was optimised by growing cells onto autoclaved 22x22 mm coverslips. Cells were washed with HBSS twice and fixed with either ice cold methanol or 4% PFA. The negative control was treated with diluents instead of biotinylated MAL. Different concentrations

(range: 2, 10, 15, 20 µg/ml) of biotinylated lectin were prepared using the manufacture's guideline and incubated for 1 h at room temperature. This was followed by the addition of fluorescent at avidin different concentrations and incubated in the dark for 30 min. The slides were stored at 4°C.

2.2.7 Validation of labelling tools using CMP

For utilising the tools in the evaluation of polySTi, they need to be validated using known inhibitor, CMP. Cells were treated with different concentrations (1 mM, 3 mM, 5 mM and 7 mM) for 24 h at 37°C in a 5% CO_2.

2.2.8 Imaging

Fluorescence images were obtained with a Leica DM2000 microscope and captured using a Leica digital camera and Leica Application Suite (LAS) software (Leica Microsystems CMS GmbH, Wetzlar, Germany).

2.3 Results

2.3.1 Kinetics of cell growth

Growth curves were established using the MTT assay for the tumour cell line panel, to determine the optimum cell seeding densities which would ensure that cells would be in the exponential phase of growth when performing the various assays used in these studies (**Figure 2.2**).

For IMR-32, cell growth was only seen at higher concentration: $5x10^4$ cells/ml and $1x10^5$ cells/ml concentrations, whereas other cell lines showed the exponential (lag) phase from Day 0.

From the data, $1x10^4$ cells/ml (DLD-1, C6-STX and C6-WT) and $1x10^5$ cells/ml (IMR-32 and SH-SY5Y) were the optimal density selected in this thesis for further analysis unless otherwise stated.

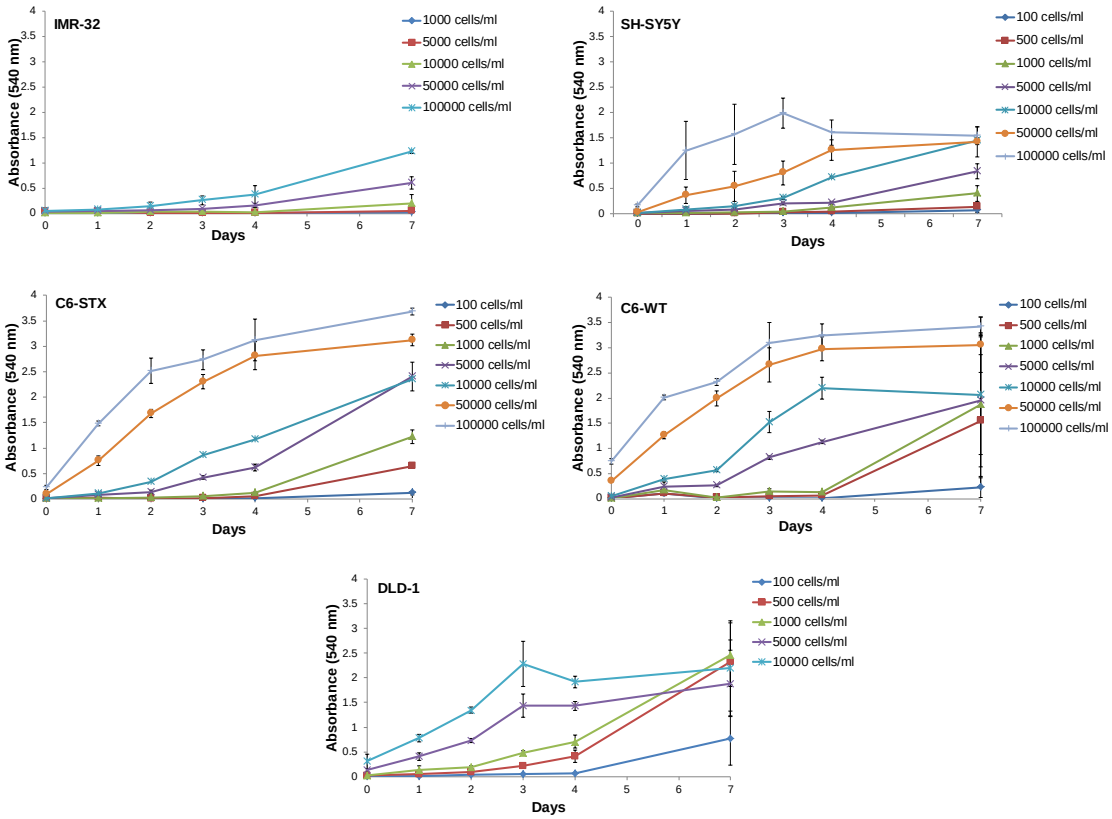


Figure 2.2: Growth curves using the MTT assay.

Cells were plated in 96-well plate on day 0 and absorbance was measured in their subsequent days. Results were expressed as absorbance mean ± SD (n=3).

2.3.2 Characterisation of polySia and NCAM expression in the cell line panel using cytochemical and immunocytochemistry techniques

Confirming the expression of polySia (using both probes mAb 735 and endoN-GFP) and NCAM in the cell line panel was carried out as previously described (**Figure 2.3**). Variables including concentration, temperature, incubation time and fixatives were investigated (**Figure 2.3**). The concentrations of endoN-GFP and mAb 735 were as previously used whereas a higher concentration of NCAM was used.

PolySia expression was evaluated using neuroblastoma cell lines IMR-32 and SH-SY5Y and polySia membranous labelling was seen in both cell lines.

Isogenic C6 rat glioma cells were also studied, one is wild-type (C6-WT) expressing NCAM only and the other was transfected with STX (C6-STX) expressing both NCAM and polySia. In this, polySia labelling was found only on C6-STX cell lines and no labelling was observed in C6-WT cell as expected.

The polySia expression pattern seen was similar in IMR-32, SH-SY5Y and C6-STX cells for both mAb 735 and endoN-GFP.

For NCAM, all cell lines highly expressed this protein apart from DLD-1 which was selected as a negative control, expressing no polySia, polySTs or NCAM and hence no labelling patterns were seen with endoN-GFP, NCAM or mAb 735 as shown in **Figure 2.4**.

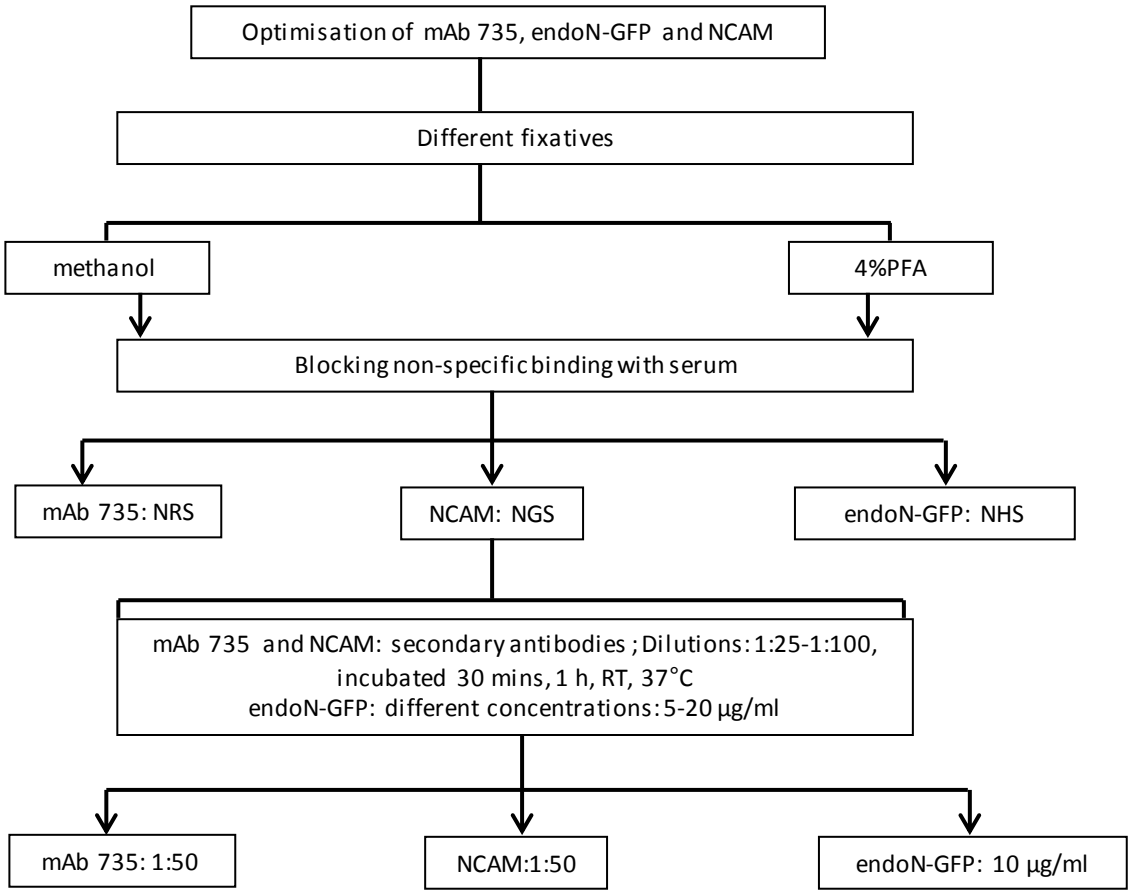


Figure 2.3: Flow-diagram of polySia labelling tools and NCAM to detect expression in the panel.

Keywords: PFA: paraformaldehyde; NRS: Normal Rabbit Serum; NGS: Normal Goat Serum; NHS: Normal Horse Serum; mAb 735: anti-polySia antibody; h: Hours; RT: room temperature.

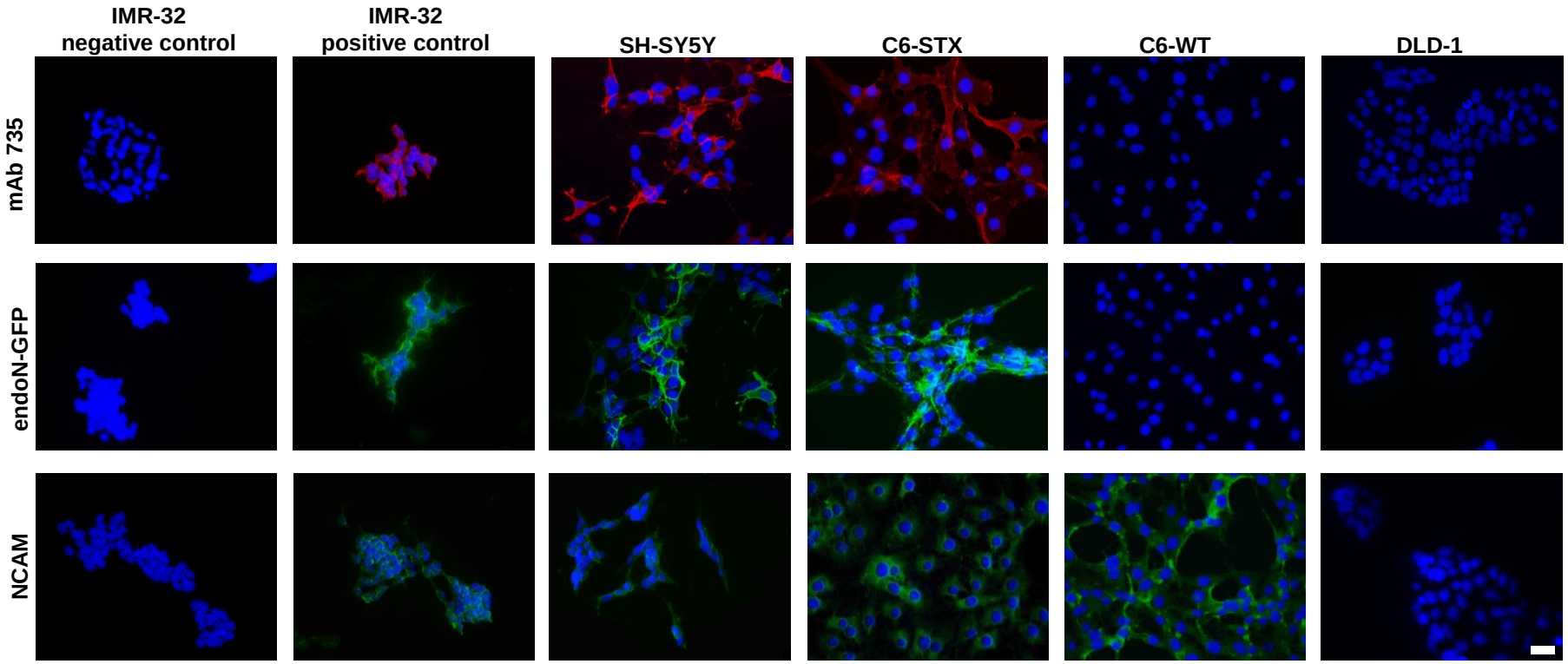


Figure 2.4: PolySia and NCAM expression of a panel of cell lines.

IMR-32, SH-SY5Y, C6-STX, C6-WT and DLD-1 cells were labelled with mAb 735, endoN-GFP and NCAM. The negative controls with only secondary antibody (mAb 735; NCAM) and serum (endoN-GFP) whereas positive controls had primary antibodies or endoN-GFP. Membranous polySia expression was seen in IMR-32, SH-SY5Y and C6-STX cells, whereas no labelling was seen in C6-WT and DLD-1 cells (polySia: red, NCAM and endo-GFP: green; counterstained with DAPI: blue). Both mAb 735 and endoN-GFP produce the same polySia labelling pattern. The images shown are representative of three independent experiments. Scale bar: 25 µm.

2.3.3 Validation of polySia labelling tools using CMP

Using immunocytochemistry, the specificity of polySia labelling tools was validated using a molecule known to inhibit polySia biosynthesis, CMP (Al-Saraireh et al., 2013).

Cells were grown on coverslips and treated with increasing concentrations of CMP for 24 h and then immunostained (section 2.2.5). Whilst it would be expected that increasing the CMP concentration might result in a decrease in polySia expression, this was only detected at high concentrations (7 mM) (**Figure 2.5**). Therefore, to confirm these findings, endoN-GFP was used. A similar result was obtained. The lack of effect of CMP was also evident in other cells, IMR-32 and C6-STX (**Figure 2.6**).

From the images, it was difficult to see any effect CMP had on the membranous expression of polySia. One possibility could be that it was difficult to distinguish between old and new membranous polySia labelling and thus no effect of CMP was seen. Therefore, the next step was to use an assay where all pre-existing expression of polySia was removed first, before challenging the cells with polySTi and assessing for re-expression of polySia.

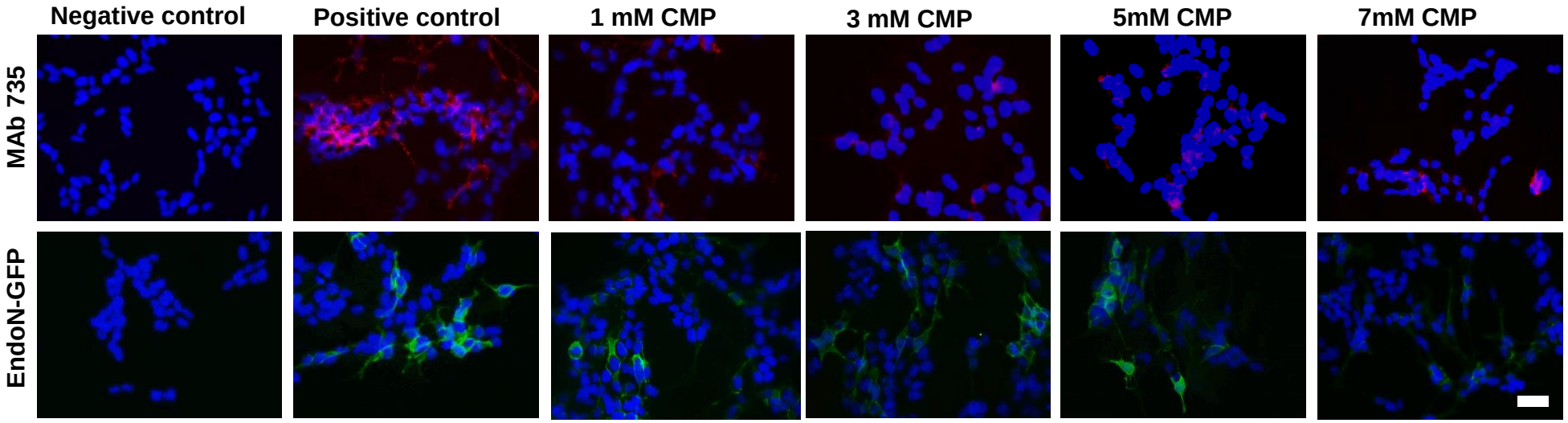


Figure 2.5: Detecting the effect of CMP on SH-SY5Y cells.

Cells were treated with increasing concentrations of CMP for 24 h and subsequently characterised using monoclonal antibody (mAb 735) and fusion protein (endoN-GFP). The positive controls (absence of CMP) contained endoN-GFP and anti-polySia antibody (mAb 735) (mAb 735: red; endoN-GFP: green: nuclei counterstained with DAPI: blue). The effect was only detected at 7mM. Scale bar: 25 µm.

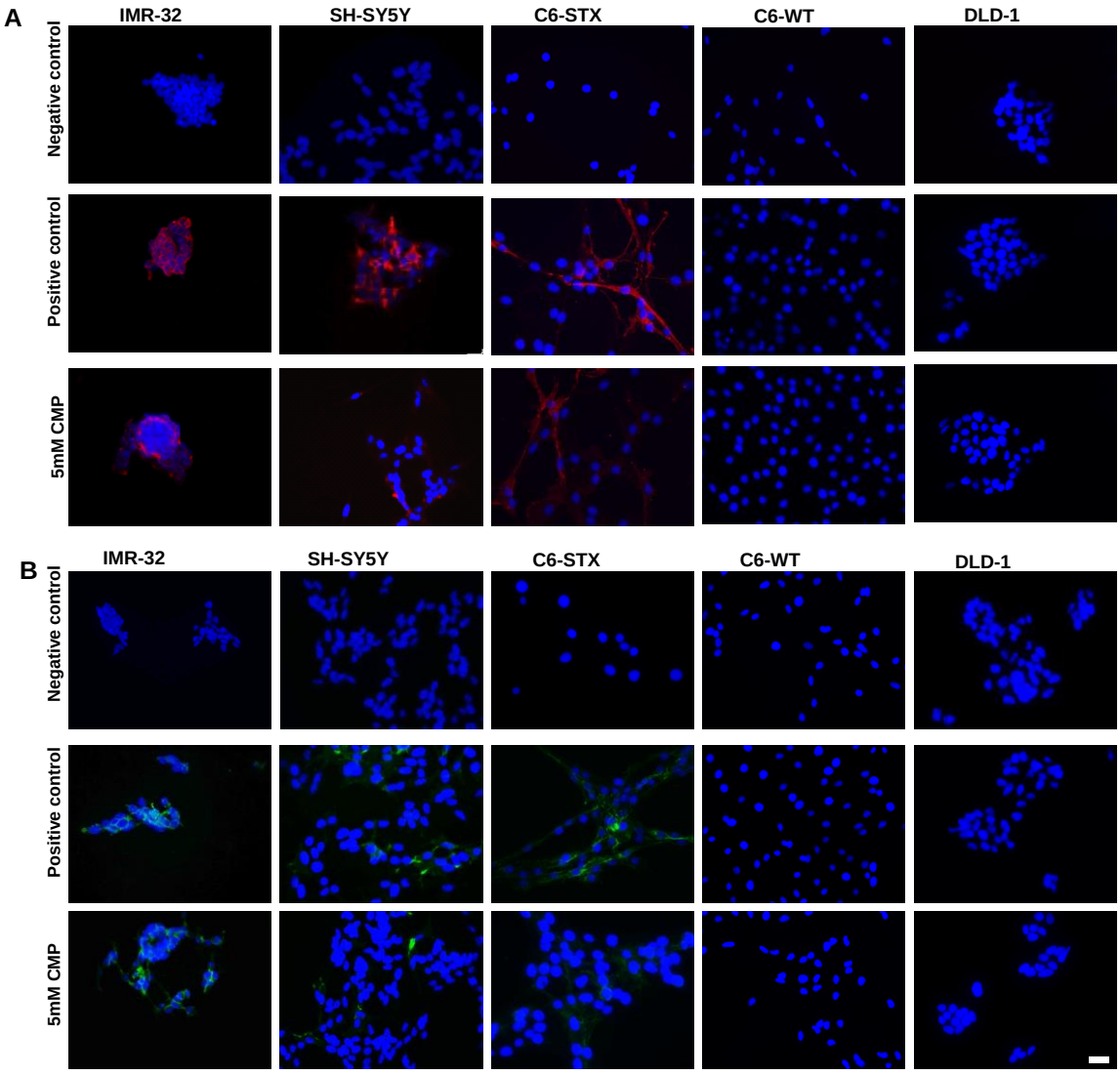


Figure 2.6: CMP has no apparent effect on polySia expression following direct treatment on cells.

Cells were treated with CMP (24 h) and characterised with mAb 735 (A) and endoN-GFP (B). The positive controls (no CMP) contained endoN-GFP and mAb 735 No obvious inhibitory effect was observed on the polySia when treated with 5 mM CMP for 24 h. (mAb 735: red; endoN-GFP: green; nuclei counterstained with DAPI: blue). Scale bar: 25 µm.

2.3.4 Optimisation of tools for sialic acid detection

As previously described, it is important to establish the specificity of polySTi for polySTs over other members of the wider sialyltransferase family. The specificity of polySTi was studied by using differential staining tools (lectins) for α-2,3 and α-2,6 sialic acids. Here, lectins MAL I, MAL II and SNA were utilised to detect expression of α-2,3 sialic acid and α-2,6 sialic acid respectively.

In initial experiments, cells were treated with different concentrations of lectins, including 2, 5, 10, 20 µg/ml and with different fixatives including 4% PFA or ice cold methanol using three different experimental conditions. MAL I and SNA was optimised using 20 µg/ml lectin concentration (**Figure 2.7** and **Figure 2.8**).

MAL II was optimised using different experimental conditions including concentration of lectin (including 10-30 µg/ml), followed by fluorescent Avidin (Texas Red or fluorescein) with 4% PFA or methanol, and shown in **Figure 2.9**.

In the optimum conditions, the expression of α-2,3 sialic acid and α-2,6 sialic acid was clearly seen on the cell surface. Then these conditions were applied in a panel and found that all cell lines were expressing α-2,3 sialic acid and α-2,6 sialic acid detected using MALI, MALII and SNA (**Figure 2.10)**.

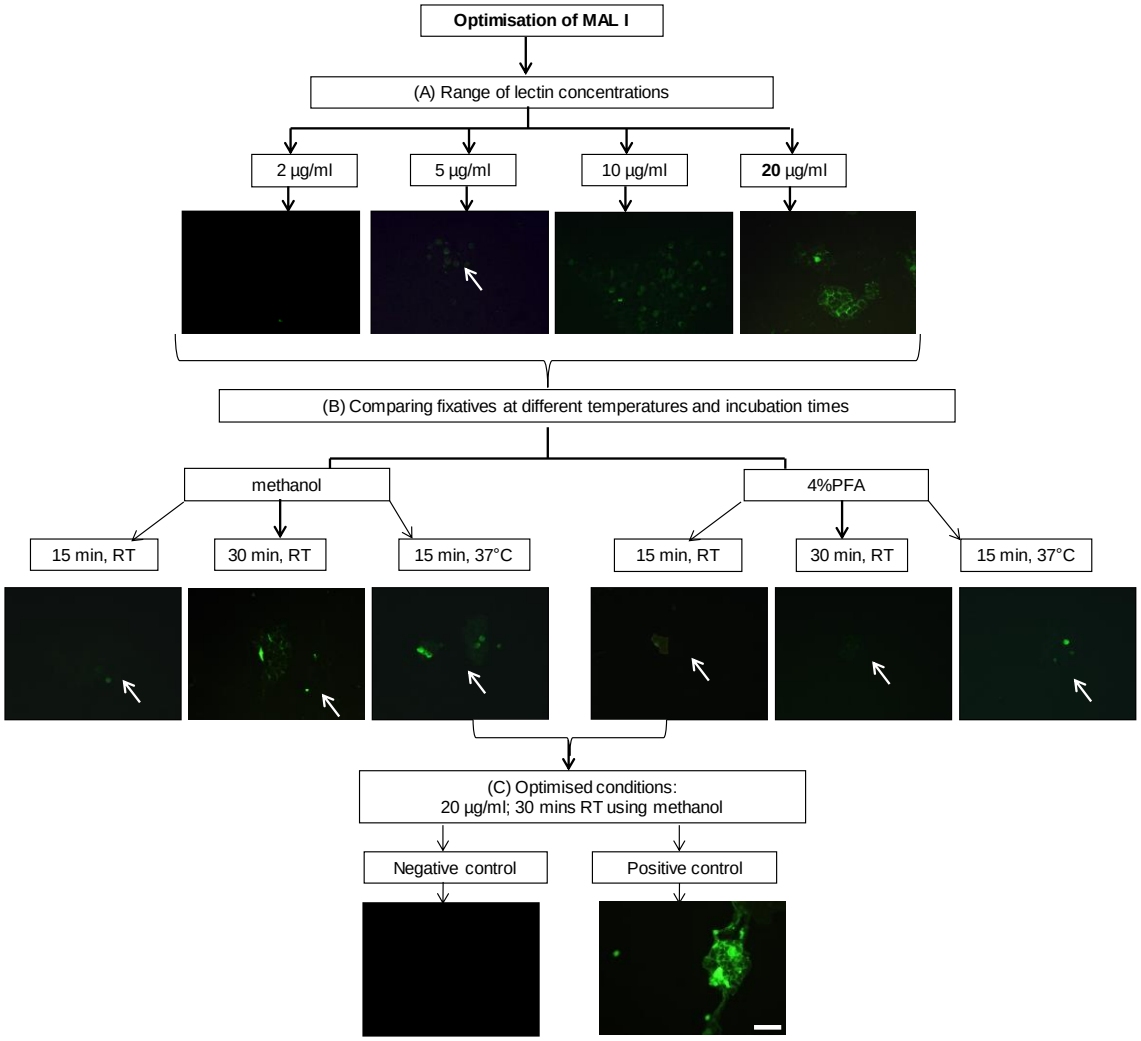


Figure 2.7: Optimisation of fluorescein labelled *Maackia Amurensis* lectin I (MAL I).

(A) IMR-32 cells were labelled with different concentrations of MAL I using different fixatives. (B) Different experimental conditions; 15 min, room temperature; 30 min, room temperature; 15 min, 37°C were applied using 4% PFA or ice cold methanol using 10 µg/ml of lectin. (C) The optimised protocol showed a representative example for IMR-32 cells. Scale bar: 60 µm.

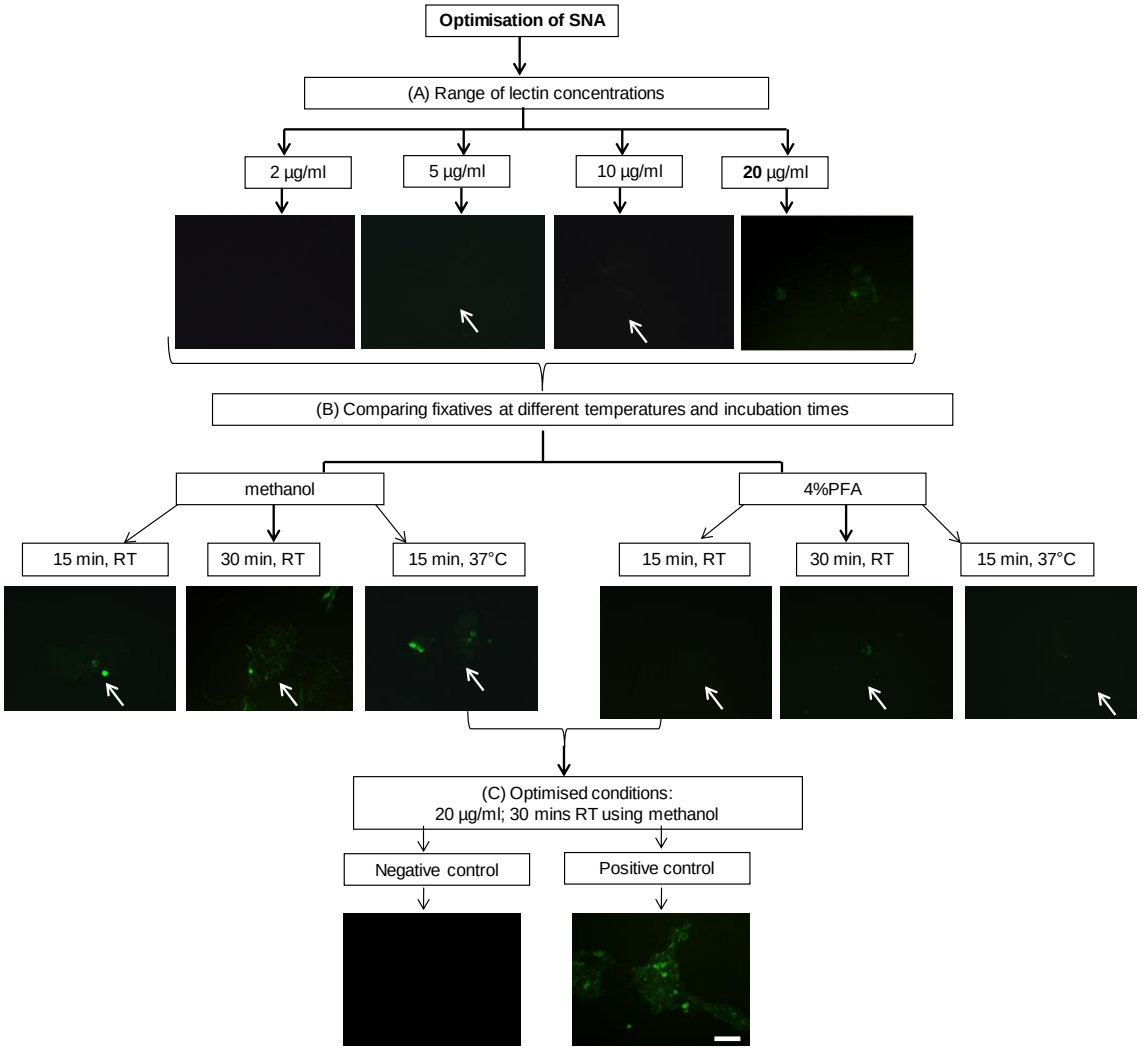


Figure 2.8: Optimisation of fluorescein labelled *Sambucus nigra* (SNA).

(A) IMR-32 cells were labelled with different concentrations of SNA. (B) Different experimental conditions; 15 min, room temperature; 30 min, room temperature; 15 min, 37°C were applied using 4% PFA or ice cold methanol using 10 µg/ml of lectin. (C) The optimised protocol showed a representative example for IMR-32 cells. Scale bar: 60 µm.

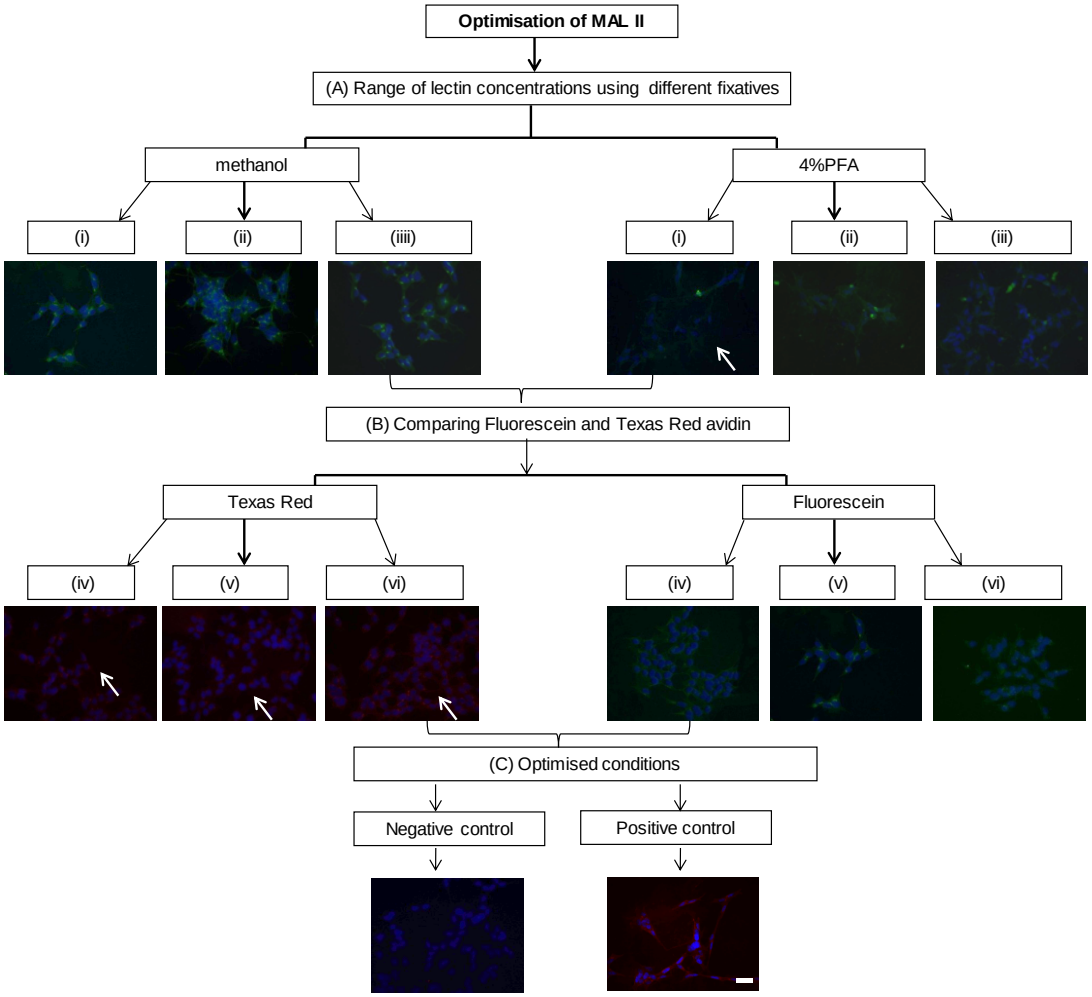


Figure 2.9: Optimisation of the Biotinylated labelled *Maackia amurensis* II (MAL II) lectins (α-2,3 sialic acid) in SH-SY5Y cells.

(A) SH-SY5Y cells were labelled with different concentrations of lectin [(i) 10 µg/ml and (ii, iii) 20 µg/ml] and avidin [(i, iii) 30 µg/ml and (ii) 15 µg/ml] using different fixatives but showed background fluorescent. (B) Comparing Fluorescein and Texas Red avidin on SH-SY5Y cells using methanol. The concentrations of lectins were (iv, v) 10 µg/ml and (vi) 20 µg/ml and avidin concentrations were (i) 5 µg/ml; (ii) 30 µg/ml and (iii) 15 µg/ml and lectin 10 µg/ml was selected. (C) The optimised protocol applied to SH-SY5Y cells showing a representative image of two independent experiments. Scale bar: 25 µm.

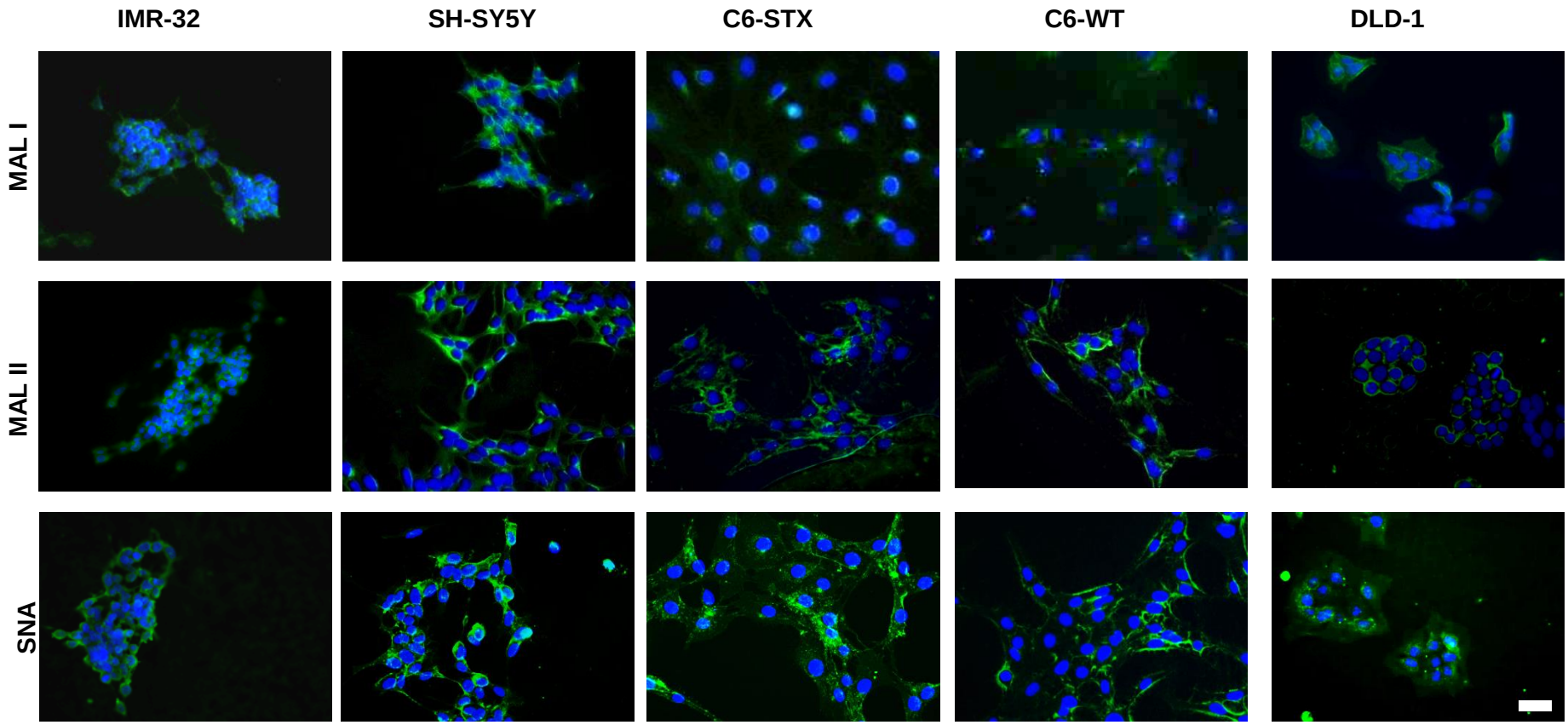


Figure 2.10: Characterisation of sialic acid expression using lectins.

Maackia Amurensis (MAL I), *Sambucus nigra* (SNA) and Biotinylated labelled lectins *Maackia amurensis* (MAL II) predominantly binds sialic acid that in linkages α,2-3 and α,2-6 in a panel respectively. Nuclei were counterstained with DAPI (blue) and each lectin binding is visualised in green. Scale bar: 25 µm.

2.3.5 Validation of lectin labelling probes with CMP

The specificity of CMP for polyST was confirmed using endoN-GFP which is an antibody-mimic probe to detect polysialylation. Cells were treated for 24 h with increasing concentrations of CMP (1 mM, 3 mM and 5 mM) and showed no effect on STs (i.e. α-2,3 or α-2,6 sialic acids) (**Figure 2.11**).

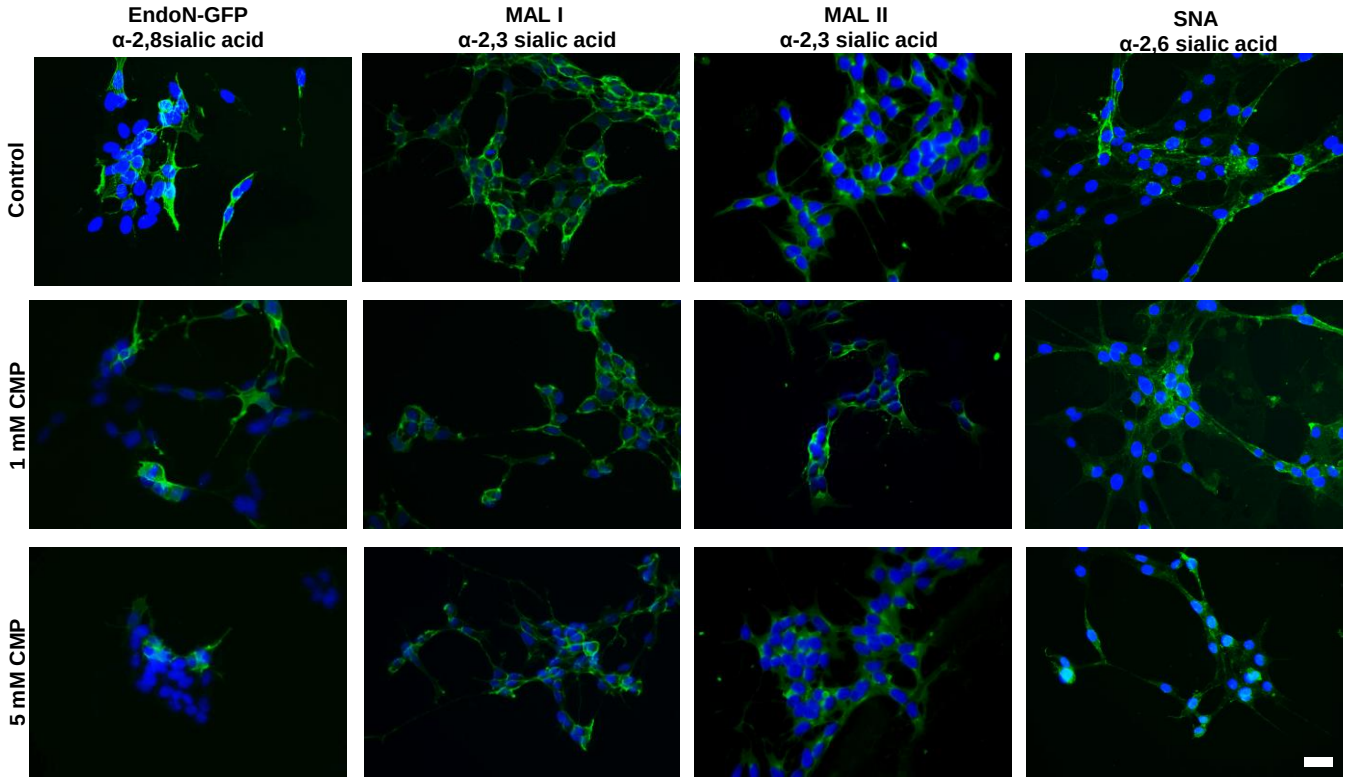


Figure 2.11: Effect of CMP on other sialic acids expression.

SH-SY5Y cells were treated with CMP for 24 h and their sialic acid expression was visualised using direct fluorescein labelled *Maackia amurensis* (MAL I), *Sambucus nigra* (SNA) and Biotinylated labelled lectins *Maackia amurensis* (MAL II) followed by avidin Texas red detecting α-2,3 sialic acid and α-2,6 sialic acids respectively and found no obvious inhibiting effect (MAL and SNA: green; and nuclei counterstained with DAPI: blue). Scale bar: 25 µm.

2.4 Discussion

The main aims of this chapter were to assemble a panel of cell lines and the detection tools of sialic acids which can be used to screen novel inhibitors of polyST.

Initially, cell growth was characterised using the MTT assay which monitors cell proliferation, in order to optimise cell seeding densities for different growth periods for use with the other assays, so that cells to be used would be in an exponential growth phase during the experimental duration. It was seen that C6-STX and C6-WT cells grew more quickly than the neuroblastoma cell lines, and seeding density was adjusted accordingly in other assays.

Tissue culture technique provides a powerful system for the discovery and validation of the new anticancer targets. In this study, naturally and non-naturally expressing positive and negative cells were selected. Since neuroblastoma was the interest of this thesis, two naturally expressing polySia cell lines IMR-32 and SH-SY5Y were selected.

A pair of rat isogenic cell lines were also utilised here. This provided the model for screening but these cells do not exist in nature. In this case C6 glioma cells were engineered to over-expressed STX, an enzyme responsible for polysialylation in neoplastic cells (Cheung et al., 2006). This model allowed us to carry out unambiguous studies of polySTi with a defined expression difference between WT and STX, without the typical confounding problem seen with unrelated cancer cell lines. The rat isogenic cell lines were extensively

used showing that C6-STX cells migrate and invade more than C6-WT cells *in vitro* indicating a useful model for screening and validating the effects of polySTi (Al-Saraireh et al., 2013, Suzuki et al., 2005). PolySia positive cells (C6-STX) invaded more than polySia negative cells (C6-WT) across the corpus callosum when injected (Suzuki et al., 2005). Other studies have shown that knock down of STX (i.e. endo-N) reduced migration caused by polySia (Eggers et al., 2011). Therefore, these studies concluded the implication of STX as an anti-polySia target and useful model for screening polySTi.

Additionally, the naturally null expressing DLD-1 cell was used as a negative for polySia, polyST and NCAM expression.

PolySia is synthesised by two polyST enzymes. During development, STX is highly expressed in embryonic brain whereas PST is expressed in the adult brain as mentioned in Chapter 1. STX is thought to be responsible for polySia re-expression in tumours as shown previously (Cheung et al., 2006, Suzuki et al., 2005). Due to a lack of antibodies recognising polyST, antibodies and probes are required to detect polySia expression. Previous studies have indicated the direct correlation of polySia with the expression of polyST enzymes and polySia cell surface expression is dependent on the polyST transcriptional level (Hildebrandt et al., 1998). For this, in this chapter different tools were utilized to assess the expression of polySia. Two methods can be used to assess the effects of polySTi on the polySia expressing cells. EndoN-GFP is a useful tool for detection of polySia expression in live cells, which binds

to polySia directly and rapidly without degrading polySia (Jokilammi et al., 2004).

Another is anti-polySia antibody mAb 735, which binds with polySia specifically (Frosch et al., 1985). By analysing tumour cell lines, the expression of polySia was validated in polySia expressing cells (i.e. IMR-32, SH-SY5Y and C6-STX), whereas no expression was detected in polySia negative cells (i.e. C6-WT and DLD-1). The cell surface labelling patterns of polySia were the same with both mAb 735 and endoN-GFP, making these tools equally specific for cell surface expression of polySia (Jokilammi et al., 2004).

The expression of polySia and NCAM were examined using anti-polySia and anti-NCAM antibodies and endoN-GFP. Cells expressing both polyST enzymes had high level of polySia expression (i.e. IMR-32), compared with SH-SY5Y which only expressed STX (Jimbo et al., 2001, Hildebrandt et al., 1998). This is consistent with previous work suggesting that both polySTs form polySia in a synergistic way (Angata et al., 1998).

A pair of isogenic C6 rat glioma cells, engineered to overexpressed STX (C6-STX) was also utilised here as polySia-positive cells, whereas C6-WT only expressed NCAM.

There was no expression of polySia or polySTs seen in C6-WT and DLD-1 cells (**Figure 2.4**) (Jimbo et al., 2001). The results in this thesis regarding polySia and NCAM expression are consistent with PCR and western blot data generated previously (Al-Saraireh et al., 2013).

In this study, mAb 735 was primarily used since it gave more consistent results compared to endoN-GFP, which was light sensitive and easily photo-bleached, resulting in low intensity signal. Therefore, for the merit of this, mainly mAb 735 was used in the functional assays (Chapter 4).

PolySia labelling tools were used to determine the effect of polySTi (Chapter 4) by utilising a known polySTi (CMP) on tumour expressing polySia cells with immunocytochemical technique. The effect of CMP was inconclusive or was difficult to interpret between old and new polySia chains at a lower concentration. But the clear effect was seen at 7 mM CMP. Therefore, a better way to determine the effect of CMP is using the recovery assay (see Chapter 3).

It is essential to determine the selectivity of polySTi as these inhibitors could affect α-2,3-ST and α-2,6-ST. Since sialic acids have extensive roles, this could be a source of off-target effects. Therefore, it was important to determine the specificity of the compounds for polySTs. CMP as a tool to evaluate the validity of the sialic acid lectin labelling approach had no differential effects on α-2,3-sialic acid and α-2,6-sialic acid.

Furthermore, this methodology needs further validation using known inhibitors of STs. For example, Lithocholic acid (LA), new and potent α-2,3-sialyltransferase inhibitor (Chang et al., 2006) and its analogue (AL10) has shown the inhibition of a-2,3-ST using human lung cancer cells (CL1-0, CL1-5) (Chiang et al., 2010).

Seeds from the legume tree seeds of *Maackia amurensis* express two Sia-specific lectins, hemagglutinin (MAH/MAL II) and leukoagglutinin (MAL/MAL I). In this study, MAL I and MAL II were obtained from Vector Laboratories assuming MAL I is MAL and MAL II is MAH. This resulted in confusion of using two isoforms for detecting α-2,3 sialic acid expressions in a panel of tumour cells. MAL has a greater affinity to bind with the sequences of trisaccharides include α-NeuAc(2,3)βGal(1-4)βGlcNAc/Glc in contrast to MAL II which has a greater affinity for tetrasaccharides (αNeuAc(2,3)βGal(1,3)[α-NeuAc(2-6)]αGlaNAc) (Imberty et al., 2000, Knibbs et al., 1991, Konami et al., 1994, Wang and Cummings, 1988) but MAL II has been used specifically for the detection of α-2,3 sialic acid.

The specificity of these lectins can vary with different experimental parameters and cell lines. This is the case with SNA, where specificity for α-2,6 sialic acid has been shown in different studies (Shibuya et al., 1987). For instance, one study mentioned SNA recognising 6-O-sulfation of galactose (β1-4 to GlcNAc linkage) using affinity chromatography (Shibuya et al., 1987, Yamashita et al., 1992).

In the present thesis, it has been concluded that these lectins are useful to distinguish between α-2,3 sialic acid and α-2,6 sialic acid from polySia.

2.5 Conclusion

In conclusion, in this chapter a panel of cell lines and appropriate detection tools has been assembled for use in further studies. The panel consists of cell lines that naturally express polySia (IMR-32 and SH-SY5Y), a null expressing natural cells (DLD-1), plus an isogenic pair of cell lines with high or low expression (C6-STX and C6-WT), and expression of polySia was confirmed using immunocytochemical and cytochemical techniques. In addition, expression of α-2,3 sialic acid and α-2,6 sialic acid was characterised using lectin differential probes.

CHAPTER THREE

3 Development and validation of functional assays for use in the screening of novel polyST inhibitors

3.1 Introduction

As presented in Chapter 1, there is strong evidence for the role of polySia in promoting tumour dissemination. This has led to the therapeutic strategy of reducing polySia expression by inhibiting its synthesis using polySTi, which is the focus of this thesis.

In Chapter 2, a panel of tumour cell lines was characterised in terms of polySia and polyST expression. The next step is the assembly of a battery of functional assays for the evaluation of polySTi. The first assay, the polySia recovery assay will be employed here to evaluate the effects of potential polySTi. This assay

monitors cell surface re-expression of polySia, and was adapted at the ICT from (Kiss et al., 1994), as described (Al-Saraireh et al., 2013). Briefly, endo-N is used to remove pre-existing polySia from the cell surface, and re-expression of polySia in the presence of inhibitor is monitored using immunocytochemistry. This strategy will be further explored in Chapter 4.

The next step is to monitor if the polyST inhibitor has an effect on cancer cell function in relation to tumour dissemination. Therefore, an appropriate assay is required to study the processes (i.e. adhesion, migration and invasion) involved in tumour dissemination. Assays which evaluate migration are already established for use in the screening of novel polySTi in-house, therefore the focus here is to investigate another facet of tumour dissemination; invasion. Invasion and metastasis are the primary characteristics of cancer-related metastatic disease, and account for 90% of cancer-related deaths (Hanahan and Weinberg, 2000). Invasion can be defined as the movement of cells through the extracellular matrix (ECM).

There are various *in vitro* invasion assays (**Table 3.1**) available for studying tumour cell invasion, including transwell invasion assays, 3D cell tracking and spheroid invasion assays. These assays are widely used as they are inexpensive, easy to handle, highly reproducible and suitable for high throughput drug screening. These techniques could be employed to study a single step of the metastatic process and the most important step is the invasion of the basement membrane (Albini et al., 2004).

In this study, transwell invasion assay (also known as Boyden chamber assay (Boyden, 1962)) has been utilised to investigate the effect of polySTi on tumour cell invasion. In this assay, two compartments are separated by a porous membrane filter, coated with a thin layer of ECM prior to cells being seeded into upper wells permitting cell invasion to the lower chamber in response to the presence of chemoattractants in the chamber (e.g. low to high serum gradient) (Albini et al., 2004, Marshall, 2011). ECM coating the membrane prevents non-invasive cells from migrating through the membrane. The number of invaded cells observed in response to chemoattractant can be counted by a light microscope. Transwells are commercially available with different pore sizes (3 µm-12 µm) or alternatively, different ECM coatings can be used with different cell types.

In this study, Matrigel coating was used, which is prepared from murine Engelbreth-Holm-Swarm (EHS) tumour, which contains laminin, collagen IV, entactin and other growth factors (Benton et al., 2014). Matrigel is a good model for ECM and coating transwells with Matrigel allowed a rapid quantification of the invasive potential of metastatic cells (Albini et al., 1987). Invasion assays have previously been employed using SH-SY5Y neuroblastoma cells treated with the synthetic Sia precursor (ManNProp; see chapter 4 for details), resulting in a reduction in the invasion ability of these cells (Gnanapragassam et al., 2014). Therefore, this assay has been applied here to evaluate the effect of novel polySTi on polySia mediated invasion (Chapter 4).

Despite the advantages described, one limitation of the transwell invasion assay is that it does not fully recapitulate *in vivo* cellular invasion, which is complicated by factors such as tumour heterogeneity and 3D invasion patterns. Therefore in order to confirm that the relevance of the results seen with the transwell invasion assay, a 3D spheroid invasion assay, which better represents the *in vivo* situation was thus employed here. In this method, spheroids of invasive cells (e.g. U87MG, C6) are embedded in different ECMs (such as collagen I, matrigel), with invasion being measured and compared to non-invasive cells after cultivation (Nowicki et al., 2008). Invasion of cells can be analysed by live imaging and quantified using imaging software (i.e. Image J). Furthermore, the spheroids can be immunostained and processed using confocal microscopy (Wolf et al., 2007). The key advantage of this assay is the movement of cells in a 3D matrix, which closely mimics invasion *in vivo*. This is significant as invasion in spheroids occurs from spheroids (cell clusters) with well-established cell-cell interactions instead of single cells, since this situation is found in cancer cells (Kramer et al., 2013). But there are also other advantages which are listed in **Table 3.1**.

Table 3.1: Comparing commonly used methods to evaluate tumour cell invasion. Taken from (Kramer et al., 2013).

	Transwell invasion assay	Platypus invasion assay	3D cell tracking	Gelatin degradation assay	Vertical gel invasion	Spheroid/monodispersed cell invasion assay	Spheroid conformation assay	Spheroid invasion assay
Chemotaxis	+	-	AD	-	-	ND	ND	-
Measurement	Cell number	Invasion area	Invasion distance	Matrix degradation area	Deepness of invasion	Cell number	NA	Invasion area
Live imaging	-	+	+	+	-	-	-	+
ICC/IHC	-	+	AD	+	+	+	+	+
Substrate	BME, Collagen I	BME	ECM	Gelatin	ECM	ND	ND	ECM
Direction of movement	Vertical	Horizontal	Any	Vertical	Vertical	Any	Any	Any
HTS	+	+	+	-	-	-	-	-
Analysis	Endpoint	Kinetics	Kinetics	Kinetics/ Endpoint	Endpoint	Endpoint	Endpoint	Kinetics

Keywords: AD: any dependent; ND: not determined; 3D: three dimensional; +: suitable; -: Not suitable; ICC: immunocytochemistry; IHC: Immunohistochemistry; BME: Basement membrane extract; HTC: high throughput screening; NA: not applicable.

3.1.1 Aims and objectives

The main aims of this chapter are to develop and validate functional assays for the analysis of novel inhibitors of polyST. These aims will be addressed by the following objectives:

- Validation of the polySia recovery assay to evaluate the inhibition of polySia using the characterised inhibitor CMP.

- Development and validation of a 2D Matrigel functional invasion assay to evaluate polySTi, again using the characterised inhibitor CMP. Confirmation of the relevance of the results seen with the 2D Matrigel assay using a 3D spheroid assay.

3.2 Materials and Methods

3.2.1 Materials

All cell culture reagents and general chemicals used are as specified in Chapter 2 unless otherwise stated. Details of the cell lines investigated in this study are given in section 2.2.2. Endoneuraminidase N (endo-N) (0.3 µg/ml) was purchased from AbCyss (Paris, France) and stored at -20°C.

3.2.2 Polysialic acid recovery assay

When cells reached 70% confluence, they were washed with HBSS and trypsinized as described above. Cells were seeded onto autoclaved 22x22 mm glass coverslips in 6-well plates. The plates were incubated overnight at 37°C in a 5% CO_2 atmosphere. All experimental steps were carried out at room temperature except where stated.

Once cells had adhered, coverslips were rinsed twice with HBSS and treated with endo-N in cell culture medium for 24 h to remove the pre-existing polySia on the cell surfaces. Cells were fixed directly after endo-N digestion to confirm polySia removal (control). Cultures with endo-N treatments were incubated with either with medium or CMP for 6 h (**Figure 3.1**).

Recovery of polySia was confirmed by incubating cultures with the complete medium only (6 h). All these groups were fixed with ice cold methanol at -20°C for 30 min. Then coverslips either stored at -20°C or immunocytochemistry was performed immediately as stated in section 2.2.5.

The slides were stored at 4°C and images captured using a Leica DM2000 optical microscope. Semi-quantified analysis was carried out by counting polySia positive cells compared with total number of cells using DAPI. Results were expressed as percentage of polySia expression relative to the untreated control and as the mean ± SD of three independent experiments.

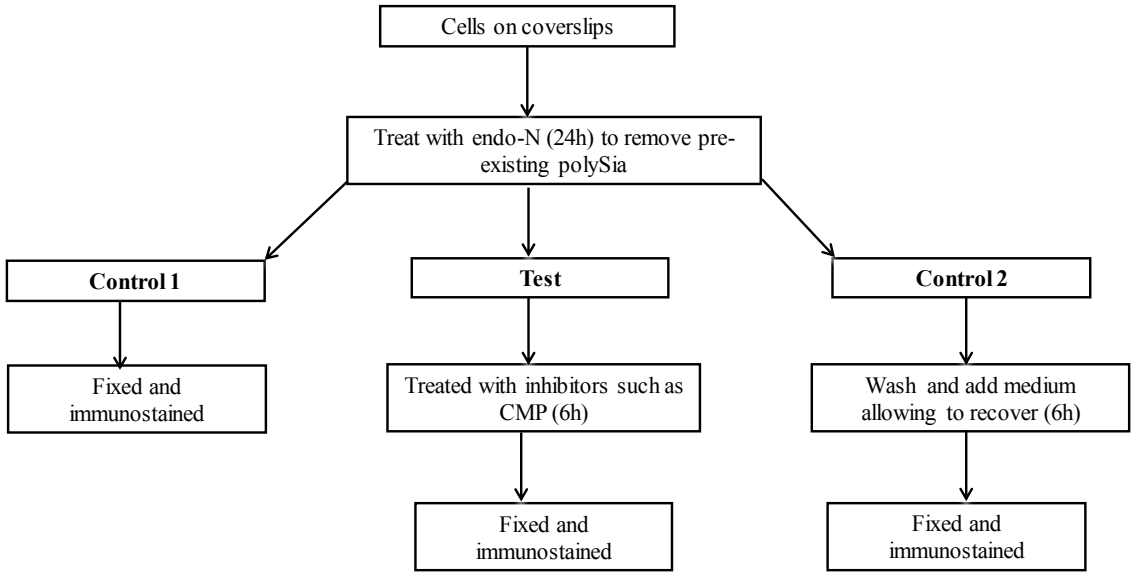


Figure 3.1: Schematic representation of polySia recovery assay.

3.2.3 *In vitro* invasion assays

In order to evaluate one of the potential effects of polySTi on tumour dissemination, two assays (the transwell and the spheroid invasion assay) were utilised to look into invasion.

3.2.3.1 Matrigel invasion assay

The transwell invasion assay was adapted from (Albini et al., 1987). Transwell chambers containing 8 µm pore-size polycarbonate inserts (Corning, USA) were coated with different concentrations of Matrigel prepared in ice cold buffer [0.01 M Tris (pH 8.0), 0.7% (w/v) NaCl] (Beckon Dickinson, UK) and incubated for 2 h at 37°C and allowed to dry.

Cells were incubated in either in 10% FBS as a chemoattractant or serum free medium. Cells were detached with trypsin or solution Accutase (Sigma-Aldrich, UK). Cell suspension (150 µl) was added to upper compartments and 600 µl of medium with chemoattractant added to the lower chamber (**Figure 3.2**).

The inserts were incubated for 24 h at 37°C in a humidified atmosphere containing 5% CO_2 allowing cells to invade the Matrigel (source of ECM).

Non-invading cells on the upper surface of the membranes were carefully removed using cotton buds, whereas the invading cells on the lower membrane fixed with 70% (v/v) ethanol and stained with Vectashield hardset fluorescent

mounting medium with DAPI onto microscopic slides and visualised under the microscope, followed by further analysis.

For quantification, images of ten random fields (20X objective lens) for each filter were taken and nuclei stained with DAPI were counted. The average of three independent experiments were taken and normalised against the total number of cells in the positive control.

Once the invasion parameters of the assay were optimised, the assay was used to evaluate the invasive properties of the cells in the presence of known polySia-interfering molecules CMP (5 mM) and endo-N (0.3 µg/ml) for 24 h.

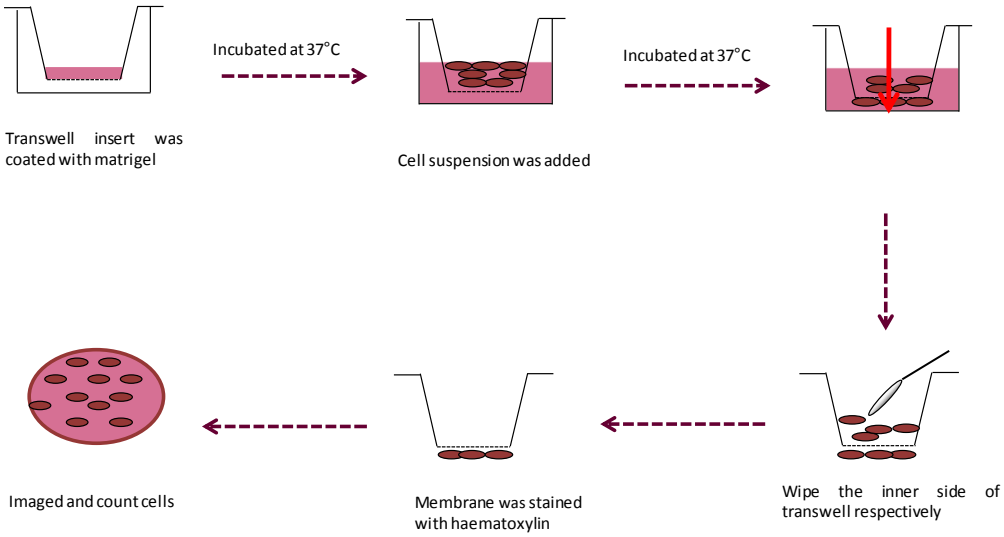


Figure 3.2: Schematic presentation of the Matrigel invasion assay.

Cells are suspended in serum free medium and added in the upper chamber. Transwell is consisting of 24 well cell culture insert with 8 µm pore size, uniformly coated with the Matrigel. Transwell were incubated for 24 h, non-invading cells removed and invaded cells were counted.

3.2.3.2 Spheroid invasion assay

3.2.3.2.1 Sourcing spheroids and test of viability using the MTT assay

A schematic representation of the spheroid invasion assay is shown in **Figure 3.3**. In this thesis, the hanging drop method is used to prepare spheroids as described previously (Del Duca et al., 2004). Different cell concentrations (i.e. 200, 1000, 10000 cells/30 µl) were prepared and drops (30 µl) placed on the inner side of the lids of a petri dish (100 mm dishes). The lid was inverted and hanging drop cultures incubated in an incubator at 37ºC. PBS was added at the bottom of the petri dish to prevent drops to evaporate since small volume was used. Cells were collected at the bottom of the drop, where they proliferate and aggregate to form spheroids.

Spheroids formed from different seeding densities were transferred to the wells of a 96 microplates and the detection of viable cells was carried out as for the MTT assay on Days 3, 5 and 7 post-seeding (section 2.2.3.3).

3.2.3.2.2 Collagen invasion Assay

In order to create a substrate for the invasion assay, type I collagen (Sigma-Aldrich) (1400 µl) is diluted with complete medium (200 µl) as described (Nowicki et al., 2008). The solution is initially acidic; therefore, 1M NaOH was added to neutralise the pH to 7.4 so that the collagen gels. 200 µl diluent was added into 8-chamber slides and slides were incubated for 30 min at 37°C incubator (no CO_2) for gelling. Spheroids were then implanted into the gel using

Gilson pipette. After gelling at 37°C, the gel was loaded with 200 µl medium and incubated for over a week at 37°C, 5% CO_2. Prior to imaging, spheroids were stained for 24 h by adding Hoechst 33342 stain as counterstain for all nuclei when treated with CMP. Images were taken using Lumascope 500 microscope and the inverted light microscopy (Nikon Eclipse TE2000-4).

Spheroid images were analysed using Image J software developed by the National Institutes of Health. In brief, the image was initially converted to an 8-bit type image and then into a binary image (black and white). The analysis dialogue was used to calculate the percentage of area covered by spheroid structure. In Excel, the percentage of invading cells can subsequently be determined from the processed images and compared with the untreated control.

3.2.4 Statistical analysis

All values are presented as mean ± SD, with each experiment repeated three times. Statistical difference between experimental groups was determined using Student's paired or unpaired *t*-test where appropriate. Differences were regarded as statistically significant when $p<0.05$ (Joshi et al., 2005).

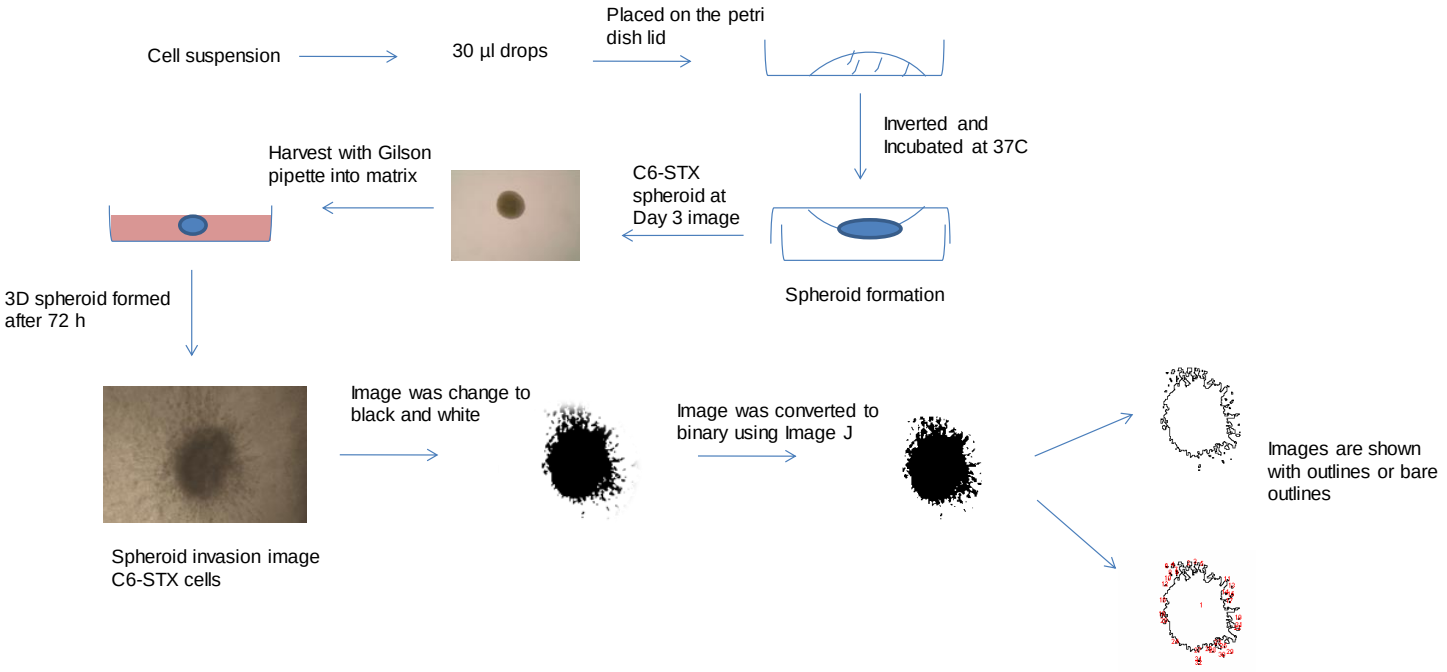


Figure 3.3: Schematic representation of spheroid invasion assay.

The basic principle involves spheroid formation using hanging drops. 30 µl of cell suspension was added on the lid of a petri dish; lid was inverted and incubated at 37ºC. Spheroids were formed at the base of hanging drops. Spheroid was transferred into 8-chamber containing matrix (i.e. collagen) and incubated for 72 h. Images were taken and analyse by Image J software.

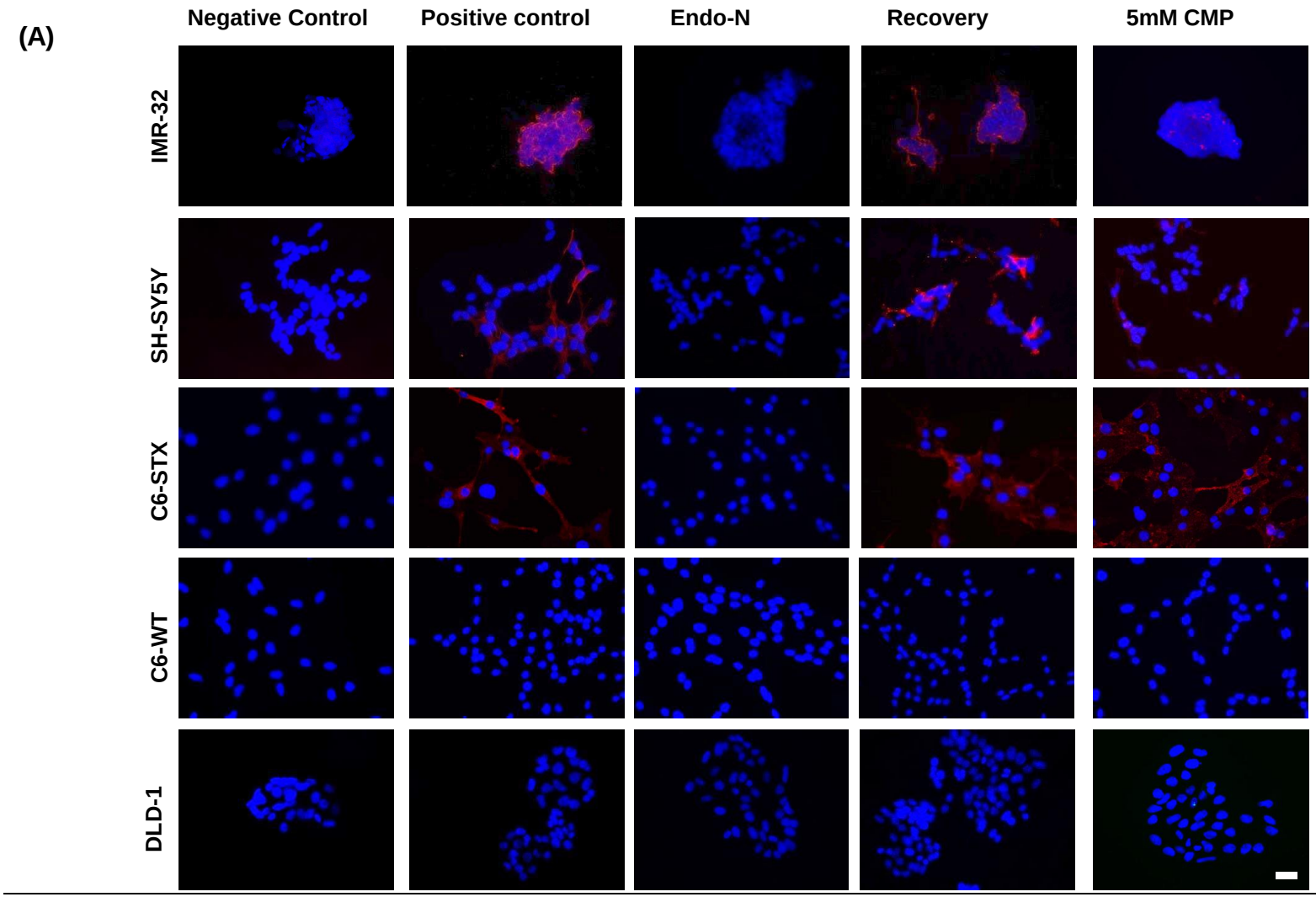
3.3 Results

3.3.1 Validation of polysialic acid recovery assay using CMP

In this assay, endo-N was used to remove pre-existing polySia cell surface expression and re-expression was monitored in the presence of polySTi CMP, with polySia detected using anti-polySia antibody (mAb 735).

PolySia-expressing cell lines (IMR-32, C6-STX and SH-SY5Y) were utilised. PolySia expression was removed using endo-N (24 h) with expression being recovered after 6 h. This was confirmed in the untreated cells (**Figure 3.4**).

CMP treatment showed a reduction in the rate of re-expression of polySia in SH-SY5Y cells. A concentration of 5 mM was selected due to persistent inhibition of polySia being observed and no cell cytotoxicity was seen. Other polySia expressing cells IMR-32 and C6-STX showed similar results. A lack of polySia expression was confirmed for the negative control, C6-WT. This result confirmed that CMP reduces polySia biosynthesis, and that the assay can be further utilised to evaluate novel polySTi.


(A)
Negative Control
Positive control
Endo-N
Recovery
5mM CMP
IMR-32
SH-SY5Y
C6-STX
C6-WT
DLD-1

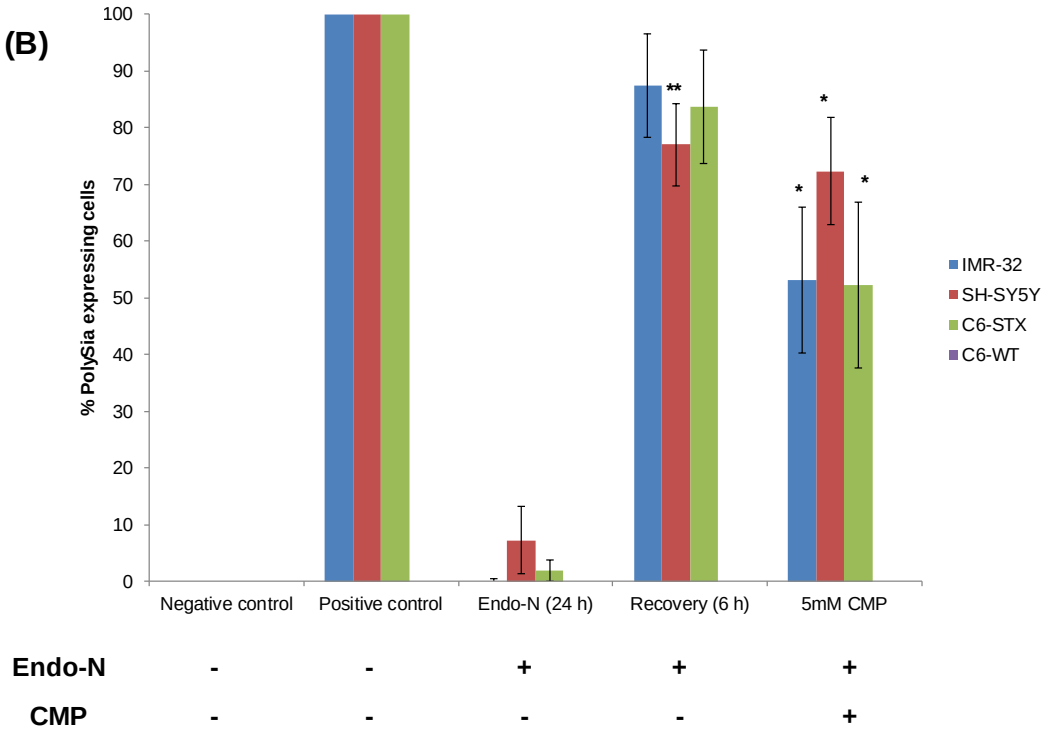


Figure 3.4: Recovery of polySia cell surface expression using anti-polySia antibody (mAb 735).

Cells were immunolabelled using anti-polySia antibody (mAb 735) followed by exposure to TRITC-conjugated secondary antibody (A). Negative control with no anti-polySia antibody, whereas positive control showed polySia expression (no CMP or endo-N). Cells were treated with endo-N for 24 h to remove pre-existing polySia expression, and allowed to recover in culture medium or treated with CMP for 6 h (polySia: red; nuclei counterstained with DAPI: blue). CMP evidently prevents polySia recovery on the cell surfaces of SH-SY5Y and C6-STX cells. The values shown are mean ± SD of 3 independent experiments (*, **$p<0.05$ to positive control) (B). Scale bar: 25 µm.

3.3.2 Validation of a Matrigel invasion assay using CMP

Studies have previously provided evidence of a role for polySia in invasion (Suzuki et al., 2005). Therefore, after polyST inhibition using CMP was confirmed to reduce polySia cell surface expression, the next step was to investigate whether polyST inhibition (and reduction in polySia synthesis) inhibits invasion. This was investigated using CMP and the Matrigel invasion assay.

Initially, the Matrigel invasion assay was optimised using different concentrations of Matrigel, cell lines, seeding densities and cell suspensions prepared in different concentrations of FBS is summarised in (**Figure 3.5A**).

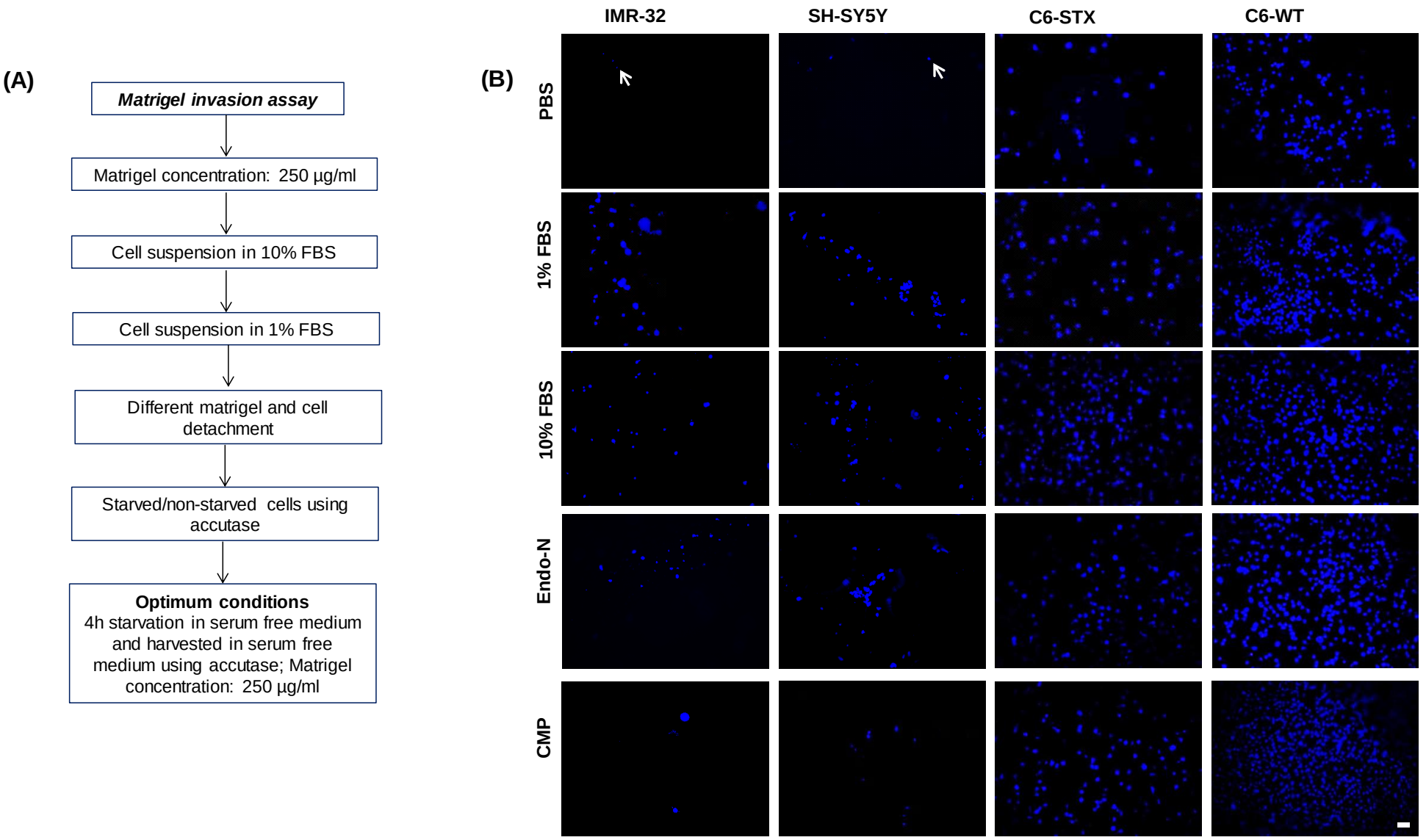
Cell invasion was also compared using starved and non-starved conditions and also using different cell detachments. It was found that starved cells invaded less in solutions containing no chemoattractant (i.e. no FBS) compared to non-starved cells. Cell starving increased the sensitivity of the cells to the chemoattractant and this resulted in reducing spontaneous invasion.

Correspondingly, more invasion events were observed using Accutase compared to trypsin in the positive control. Accutase was selected for cell detachment since trypsin can be damaging to cells or receptors, which in turn could affect cell invasion.

Furthermore, C6-STX cells were starved for 4 h in serum-free medium and cell suspension were additionally prepared in serum-free medium and detached

using Accutase. This resulted in a large number of cells invading in 10% FBS (positive chemoattractant) compared with 1% FBS and HBSS (negative chemoattractants) (**Figure 3.5B**).

CMP and endo-N treatment significantly reduced the invasion of IMR-32, SH-SY5Y and C6-STX cells compared to the untreated positive control (10% FBS); no effect of endo-N or CMP was observed for C6-WT invasion (**Figure 3.5C**).


(A)
Matrigel invasion assay
Matrigel concentration: 250 µg/ml
Cell suspension in 10% FBS
Cell suspension in 1% FBS
Different matrigel and cell detachment
Starved/non-starved cells using accutase
Optimum conditions
4h starvation in serum free medium and harvested in serum free medium using accutase; Matrigel concentration: 250 µg/ml
(B)
IMR-32
SH-SY5Y
C6-STX
C6-WT
PBS
1% FBS
10% FBS
Endo-N
CMP

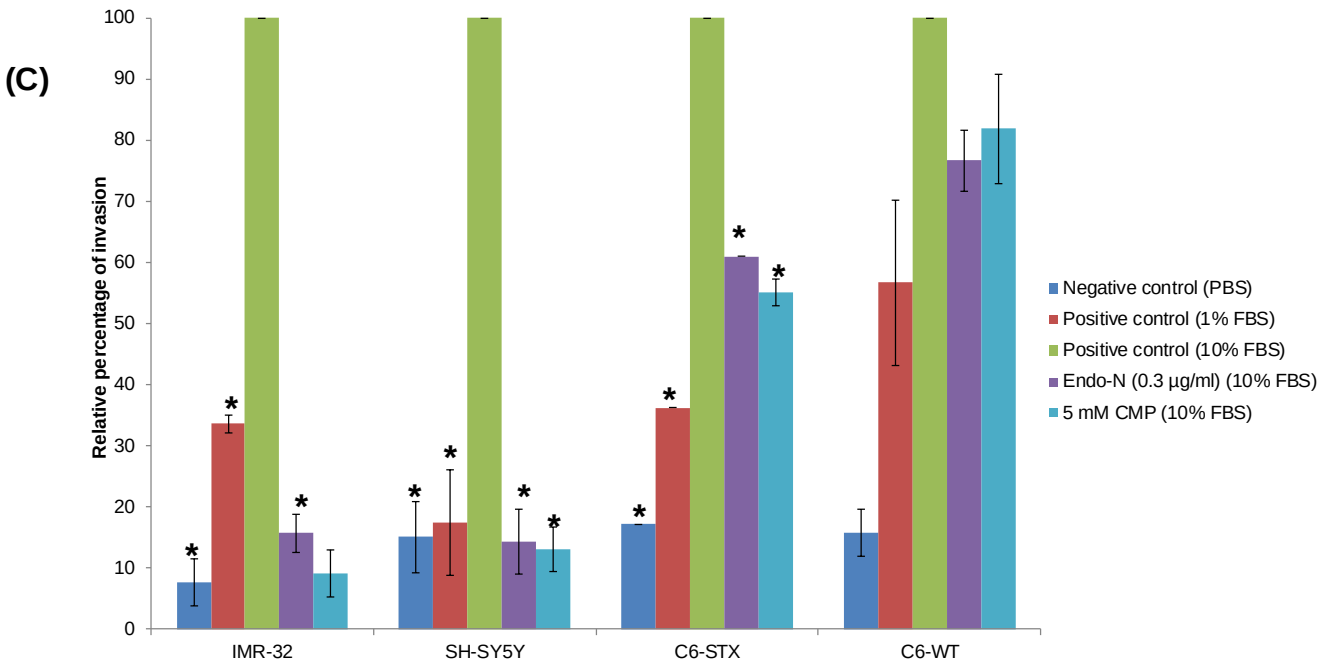


Figure 3.5: Optimisation and validation of the Matrigel invasion assay.

(A) Flow diagram of the invasion assay optimisation. The optimisation begins by investigating the effect of different concentrations of serum, Matrigel and cell densities. (B) Represented view of cell invasion. The cell suspension was added onto Matrigel-coated transwells inserts; invaded cells were stained with DAPI and counted using fluorescence microscopy. (C) The percentage of cells invading the insert was calculated as percentage of invasions. The quantitative evaluation of invasion assay showed a higher rate of cell invasion in 10% FBS. The data presented as mean ± SD of three independent experiments. The significant reduction in invasion was found compared to the positive control (10% FBS) for the endo-N and CMP in polySia expressing cells (IMR-32, SH-SY5Y and C6-STX).

3.3.3 Establishment and validation of the spheroid invasion assay using CMP

3.3.3.1 Formation of spheroids using the 'hanging drop' method

To establish the 3D invasion assay for this study, we required the following desirable features: (i) single spheroid formation (ii) high reproducibility (iii) ability to image and (iv) simple analysis. The hanging drop assay (Del Duca et al., 2004) has all these features and was applied to form spheroids.

3.3.3.2 Evaluation of effect of CMP on invasion of C6-STX spheroids using a spheroid invasion assay

C6-STX cells formed compact spheroids. The aggregate become visible after 24 h to 48 h as shown in **Figure 3.6**.

Different cell densities in the hanging drops (100, 1000, 10000 cells/drops) were used and an increase in the pattern was found with the increase in concentration and time. Even after using different seeding densities, spheroids had similar growth trends as measured by the MTT assay (**Figure 3.6**). Furthermore, the doubling time of spheroids is more than the monolayer cells (**Figure 3.6**), signifying that the growth properties of spheroids are a better representation of *in vivo* tumour growth properties.

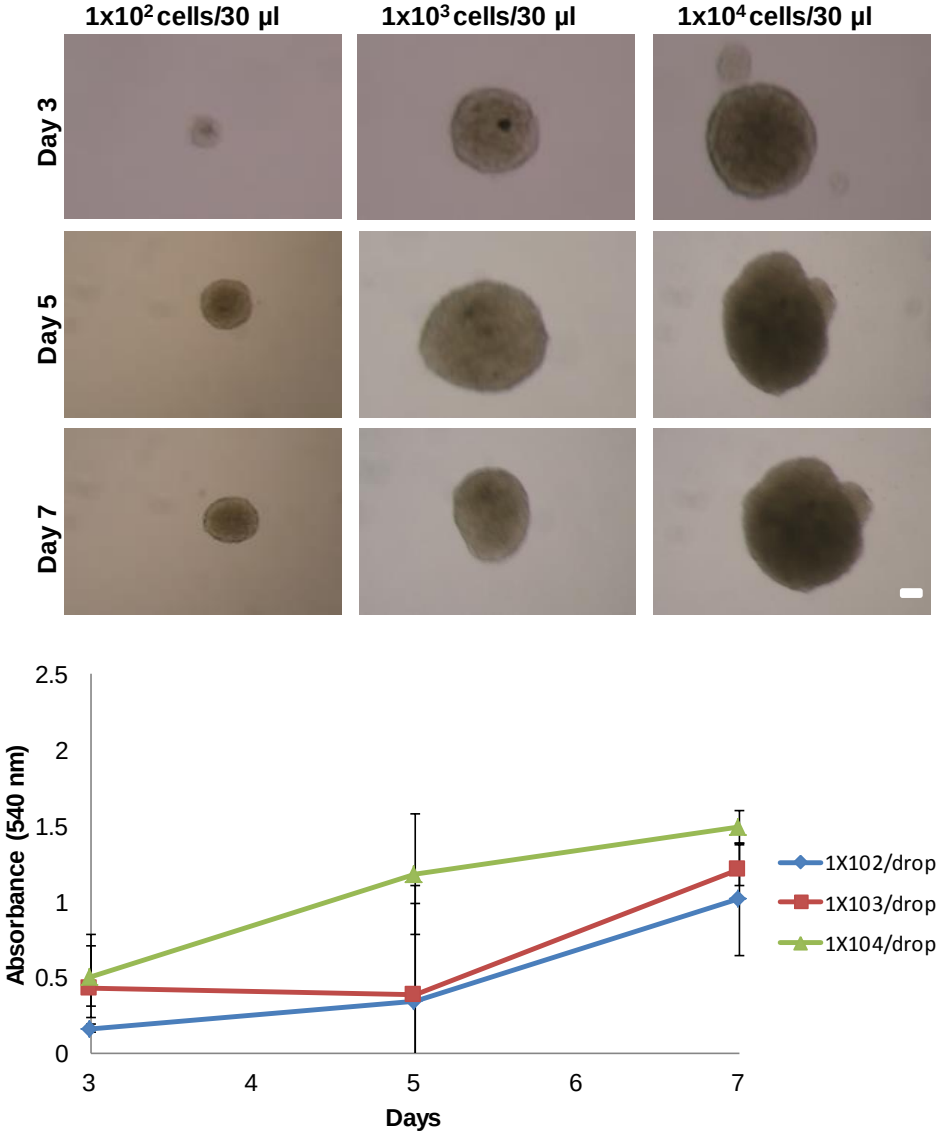


Figure 3.6: C6-STX spheroid.

Spheroids are formed and images were taken (showing Day 3, 5, 7 spheroids). The MTT assay was carried out on the spheroids on days 3, 5 and 7. The data are represented as mean ± SD of three independent experiments. Scale bar is 20 µm.

The spheroids formed by hanging drops were then transferred into collagen as shown in **Figure 3.7**. Invading spheroid images were then taken and found C6-STX cells invaded in the collagen.

The spheroid invasion assay was validated with the known polySTi, CMP. Spheroids treated with CMP (1, 3 and 5 mM) showed a slight reduction in invasion at Day 3 compared to control (untreated).

For a better visualisation of the effects, spheroids treated with CMP were also stained with Hoechst dye and similar results were observed to those described above.

Comparing 2D and 3D invasion results, it was seen that CMP had a similar effect on reducing C6-STX invasion (**Table 3.2**). Therefore, the 3D invasion assay findings validate the 2D invasion assay to be further utilized in Chapter 4.

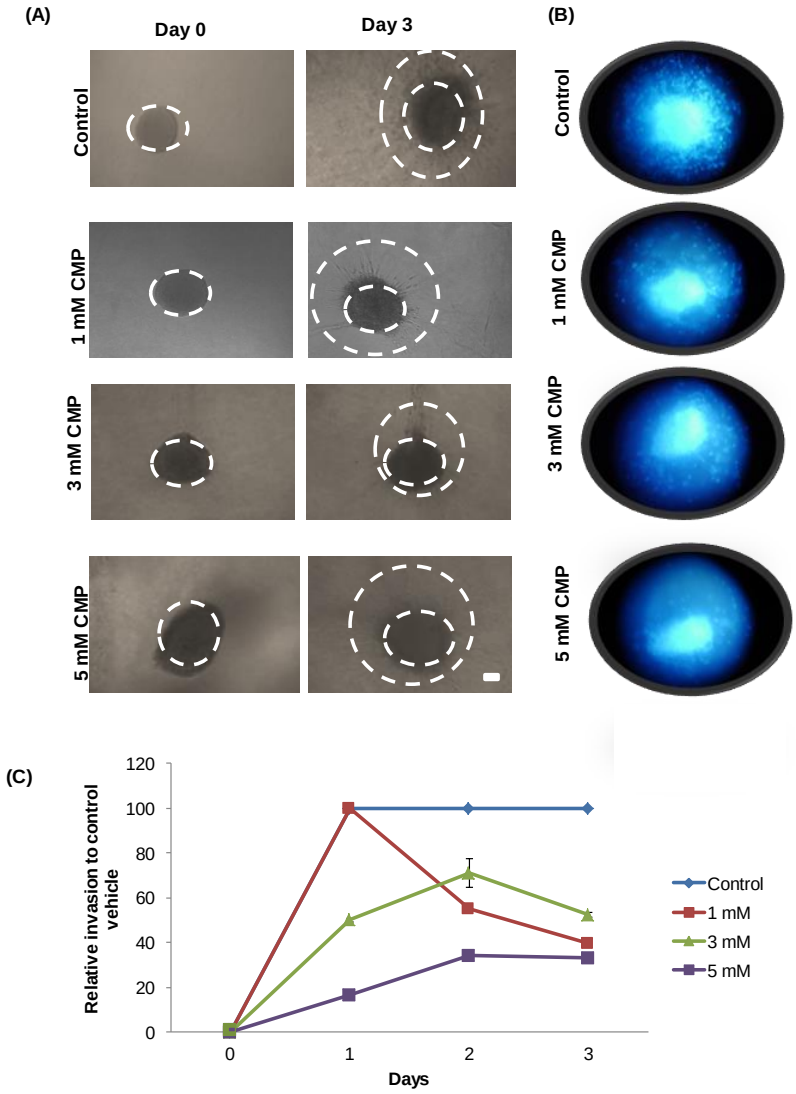
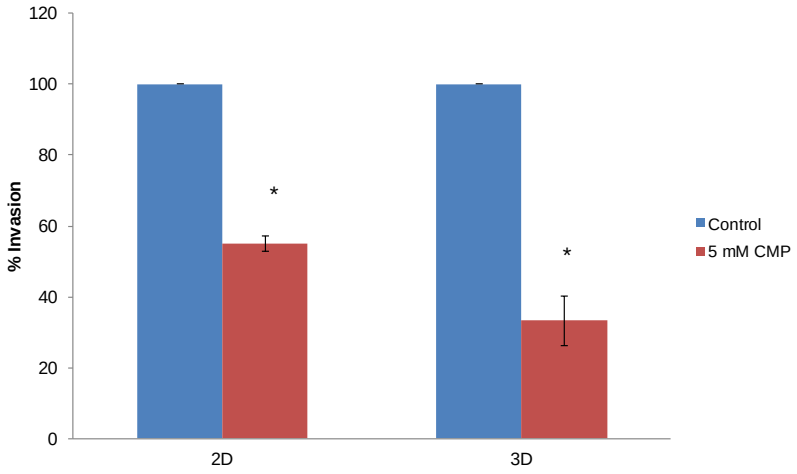


Figure 3.7: C6-STX spheroid invasion in collagen.

3D invasion assay of C6-STX (A) in a 3D collagen matrix. Spheroid invasive distance (white dotted lines) was depicted on the spheroid. (B) Viewing spheroid invasion with Hoechst 33342 stain. (C) Quantitative analysis of invasion after Day 1, Day 2 and Day 3 treated with CMP. White dotted lines indicates cell invasion pattern. Scale bar is 40 µm. The data are represented as mean ± SD of three independent experiments.

Table 3.2: Validating 2D invasion assay result with 3D invasion assay.

Parameters	2D invasion assay	3D invasion assay
Seeding	Cells added into transwell	Cells grow into spheroids and embedded into collagen
Treatment	CMP	CMP
Quantification	Invading through pores were calculated	Invading area was calculated
Results		
Conclusion	Both assay showed similar effect of CMP reducing C6 invasion.	

3.4 Discussion

The main aims of this chapter were to develop and validate assays (i.e. the recovery assay and 2D invasion assay) that can be employed in screening the novel inhibitors of polyST.

In Chapter 2, CMP was used as a known polySTi but no consistent effect was detected using the direct fluorescence method. Since, it was difficult to distinguish between the new and old growth of polySia using the direct method. Therefore, to overcome this problem, an alternative *in vitro* experimental approach was adopted to screen for modulation of polySia expression.

In this assay, recovery of polySia expression was monitored after removing pre-existing polySia expression from the cell surface using endo-N before compound treatment (Kiss et al., 1994). Here, the observed re-expression of polySia on the cell surface is due to novel biosynthesis, and comparing the levels where polySTi has been administered to untreated cultures, will give an indication of the efficacy of the polySTi. Cells treated with CMP showed a clear reduction in polySia recovery expression whereas in untreated cells, an almost comparable level of polySia was reached in polySia positive cells. Confirmation that this decrease in expression is due to STX inhibition was provided by the use of isogenic cell lines, compared with no inhibition observed with the C6-WT, which does not express this enzyme.

To-date, cancer therapies have proved unable to reduce tumour dissemination in clinical trials (Friedl and Wolf, 2003). Therefore, further understanding and targeting the molecular mechanisms involved in tumour dissemination,

especially migration and invasion could potentially lead to the development of new treatment strategies. The role of polySia in tumour dissemination of various tumours has been reviewed in Chapter 1. It is therefore very fascinating to target this polySia re-expression, as in-house data showed that CMP caused a delay in polySia expressing cell migration in a concentration-dependent manner (Al-Saraireh et al., 2013). Therefore, the next step would be to study the effect of polyST inhibitors on another feature of tumour dissemination, i.e. invasion, since many studies have correlated polySia expression with invasion (Scheidegger et al., 1994, Suzuki et al., 2005).

In this study, the Matrigel invasion assay was selected. Invasion of tumour cells requires movement through the basement membrane. Since Matrigel is a reconstituted basement membrane, this assay has the advantage over other invasion assays in mimicking closely the physiological barrier that cancer cells have to invade in order to disseminate (Albini et al., 2004). This assay allowed a rapid quantification of cell invasion (Albini et al., 2004). Through extensive optimisation of the invasion assay parameters, namely Matrigel concentrations, cell lines, cell seeded densities and FBS concentrations; the Matrigel assay was developed and validated using CMP and endo-N.

Several technical issues were encountered in attempting to establish the assay. These included a lack of differential between negative and positive controls. This was overcome by altering protocol to include serum starvation. This prevented spontaneous invasion caused by invading cells towards chemoattractant and prepared cells for optimal response to the chemotactic

agents. This is consistent with previous studies showing the serum dependency of cancer cell invasion (Reshkin et al., 2000).

Initially, trypsin was used for cell detachment but using Accutase, an enzyme containing the mixture of proteolytic, collagenolytic and DNAse activities has improved the efficiency of invasion. Trypsin is a protease which could have damaged cells or receptors results in either inhibiting or reducing the migration ability of cells. In addition, under-trypsinisation caused cells to clump together whereas over-trypsinisation cleaves adhesion molecules (including integrins) which are essential for migration and invasion (Eccles et al., 2005). With Accutase there is a gentle cell detachment, which mimics trypsin and collagenase action together and does not require cell neutralisation. In this study, the Matrigel invasion assay was optimised using C6-STX cells, as previous studies indicated they were more invasive due to polySia compared to C6-WT (Suzuki et al., 2005). SH-SY5Y, C6-STX and C6-WT cells were selected for further screening. Cells (i.e. IMR-32) invaded in clumps into the transwell were difficult to analyse and hence were not selected.

Here, polySia expression was modulated in two different ways: first with CMP, which is a polyST competitive inhibitor reducing polySia synthesis and thereby inhibiting invasion. The other is endo-N, which enzymatically cleaves polySia specifically, which leads to reduction in invasion. A significant reduction in the percentage of invading cells was observed when treated with CMP or endo-N, demonstrating the role of polySia in the invasion of polySia-expressing tumour cells. To our knowledge, this is the first study demonstrating that reduction in

cell-surface polySia through polyST inhibition leads to modulation of tumour cell invasion.

A spheroid invasion assay was also employed here to validate the results of the matrigel invasion assay. Having a 3D structure, this assay better mimics the 3D cell interactions seen between tumour cells clinically than models based on using 2D monolayers (Del Duca et al., 2004). There are many methods available to produce spheroids. Each technique has its own strengths and weaknesses in terms of spheroids size, shape and structure. Spheroids can be formed by coating round bottom plates with agarose (Friedrich et al., 2009), poly-HEMA (Ivascu and Kubbies, 2006) and by using the hanging drop method (Del Duca et al., 2004). Spheroids produced by hanging-drops were employed here.

The optical cell density and sedimentation times required for hanging drop cultures of all cell lines used was determined as a part of this study. It was also found that for the hanging drop cultures, the important parameter is cell concentration; this affects cells following their ability to form aggregates. This method proved to be a reproducible approach for the implantation of spheroids. In addition to the control of cell density and spheroid size, this method is more effective to study the invasive properties of tumour cells. Different cell densities were used to form spheroids of different cell lines so that spheroids have the size range of 300 to 500 µm in diameter, which were considered suitable starting points for performing the experimental studies (Vinci et al., 2012).

Spheroids produced from the hanging drop method were visible after 24 h and when embedded in collagen and showed invasion in a typical sun-burst pattern. Previous studies have shown that cells with a lower metastatic potential grow in tight clumps and cells with highly metastatic produce a star-like appearance, allowing a comparison of the morphology of the tumour cells and their malignant and invasive properties. For example, breast cancer cell lines such as MCF-7 forms clumps whereas MDA-MB-231 cells invade in 3D Matrigel culture to give a similar star like appearance (Kenny et al., 2007, Benton et al., 2014).

It is well understood that imaging spheroids with an optical microscope was difficult due to high levels of scattering of light on spheroids. The scattering of light on spheroids gave bright images, which were difficult to analyse using the Image J software. The addition of the spheroids into the 8-chamber wells was also problematic; either loss or clusters occurred, causing difficulty in analysing the pattern of invasion. Other challenges associated with the use of spheroids additionally included maintaining the uniform shape of spheroids, spheroid formation using small cell number and qualitative and quantitative analysis of spheroids with drugs resting (Mehta et al., 2012).

The hanging drop method was employed here to form spheroids, which is generally a quick and simple approach. But limitations include extensive labour and to maintain spheroids was time consuming (Mehta et al., 2012). Alternatively, spinner flasks could be applied, in which spheroids form via continuous spinning of cell suspensions. This technique is suitable for long term culturing but special equipment is required and non-uniform spheroids are

produced (Mehta et al., 2012). The uniform and reproducible size of spheroids is critical to study the cell behaviour and drug efficacy (Mehta et al., 2012).

Spheroids are embedded into ECM hydrogels including collagen, acting as a physiological model to study metastases (Del Duca et al., 2004, Nowicki et al., 2008). NCAM and other ECM proteins are known to be highly expressed in spheroids of neuroblastoma and glioma cells indicating its importance in both cell-cell and cell-matrix interactions (Jung et al., 2013). NCAM belongs to the immunoglobulin family, as described in Chapter 1 and would play a role in reinforcement of spheroid structures by increasing cell-cell organisation. ECM molecules (i.e. collagen I and IV, laminin and fibronectin) will initiate the formation of spheroids from C6 cell clusters and this is consistent with another study carried out on glioma BT4C spheroids, which enhanced the biosynthesis of the ECM (Jung et al., 2013, Bjerkvig et al., 1989).

Both 2D and 3D invasion assays allowed measurement of the invasion of the cells and validation was provided using CMP (**Table 3.2**). In both assays, CMP had significant effects on inhibiting invasion in the C6-STX cells. Therefore, the 3D-assay confirmed and validated the results of 2D invasion assay, giving confidence to progress with the 2D assay in further work to evaluate novel potential polySTi in Chapter 4.

3.5 Conclusion

In this chapter, *in vitro* assays were established and validated to study the effects of polyST inhibition on polySia cell surface re-expression and tumour cell invasion (2D and 3D). Thus, these assays could subsequently be used to evaluate novel polySTi in the next chapter.

CHAPTER FOUR

4 *In vitro* pharmacological evaluation of novel small molecule inhibitors of polyST

4.1 Introduction

In the previous chapters, the assembly of a panel of cell lines with a range of polySia expression levels, and the development and validation of the polySia recovery assay along with assays to assess invasion have been described. Together, these provide the tools to screen novel polySTi.

Elevated expression of polyST enzymes, particularly STX (Cheung et al., 2006), has been reported in certain pathological process including tumour invasion. Thus, inhibition of polySTs is potentially useful as an anti-tumour strategy. One approach to polyST inhibition is the so-called 'biochemical engineering' approach, whereby unnatural Sia analogues are introduced into the Sia

biosynthetic pathway (see review (Keppler et al., 2001)). The enzymes in the Sia biosynthetic pathway (**Figure 4.1**) are permissive to these modifications: *N*-acetyl D-mannosamine (ManNAc) is the physiological precursor of *N*-acetylneuraminic acid, but *N*-acyl-modified mannosamines are efficiently converted into *N*-acyl-modified Sia in *in vitro* and *in vivo* conditions. This results in unnatural sialosides expressed on the surface of the glycocalyx (Mahal et al., 2001, Bork et al., 2007, Keppler et al., 2001, Kayser et al., 1992), where depending on the cell type, this replaces 10-85% of natural physiological Sia (for details, see review (Keppler et al., 2001). This 'engineering' also provides a new tool to explore the diagnostic and therapeutic applications of *N*-acyl side chains of Sia listed in **Table 4.1**.

Previous studies have shown the application of novel synthetic *N*-acyl-modified *D*-mannosamines, such as ManNProp, which interferes with NCAM polysialylation by changes driven by the polyST enzymes (Mahal et al., 2001, Horstkorte et al., 2004a). Conversely, other studies have shown that mammalian cells readily utilised ManNProp and *N*-butanoyl mannosamine (ManNBut), but they act as effective inhibitors of polySia synthesis, through inhibition of polyST (Mahal et al., 2001, Liu et al., 2000). In this thesis, this approach has been used to study the effect of novel small molecules designed as polySTi in the ICT on polySia tumour expression and invasion.

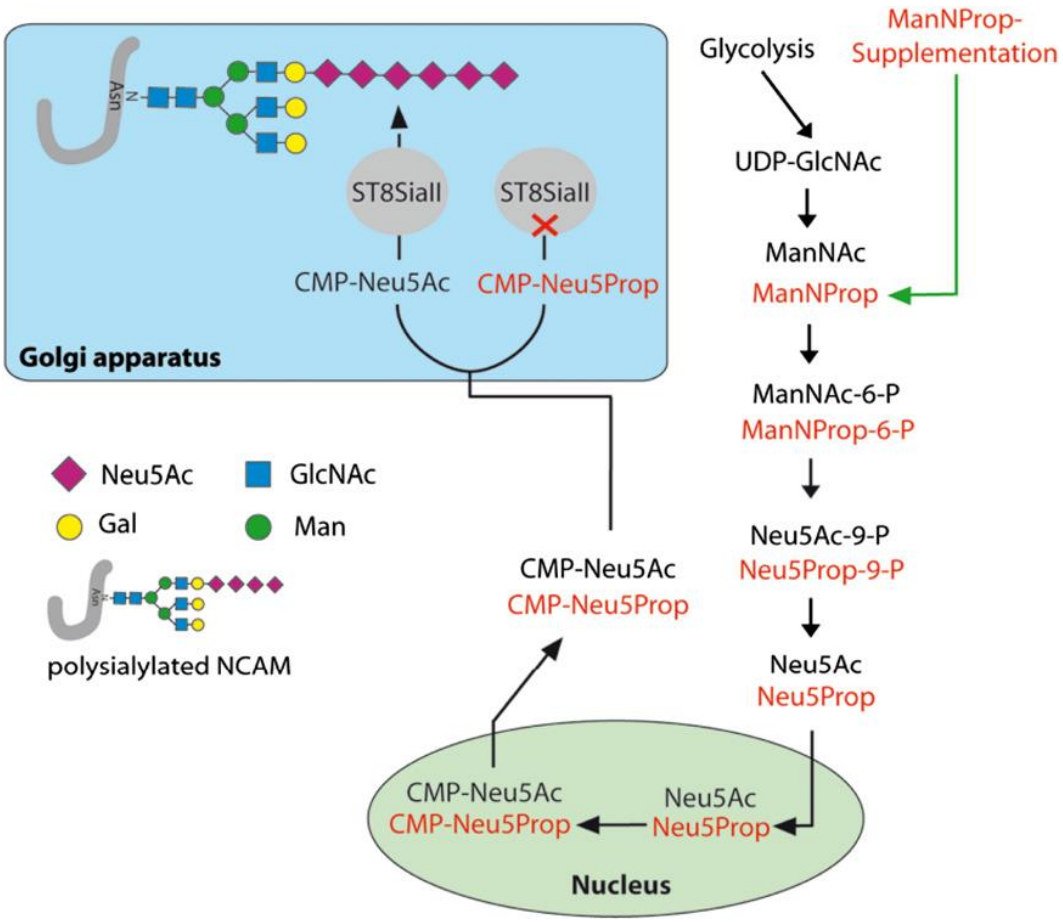


Figure 4.1: Representation of the unnatural sialic acids pathway.

Sialic acid synthesis starts in the cytosol. Natural ManNAc can be substituted with unnatural mannosamines (e.g. ManNProp), which has no effect on other enzymatic reactions. These synthetic precursors are processed by the Sia pathway in the cytosol and are incorporated into cell surface glycoconjugates. In the nucleus, the end product is formed (CMP-Neu5Ac (Sia) /CMP-Neu5Prop), which transferred to the Golgi apparatus where sialyltransferases are present. Taken from (Seifert et al., 2012).

Table 4.1: Biological implications of the incorporation of unnatural sialic acids.

Precursor	Cell type	Biological effect	Reference
		Viral uptake inhibition	
ManNProp **ManNBut** **ManNPent**	Human B-lymphoma (BJA-B)	Loss of virus binding (4 to 8 fold) and reduction (10 to 20 fold) of Lymphotropic papovavirus	(Keppler et al., 1995)
		Neural cells stimulation	
ManNGcPa	Rodent neuroblastoma/glioma (NG108-15) Human T-lymphoma (Jurkat)	Myelin-associated glycoprotein binding to NeuGc positive cells abolish	(Collins et al., 2000)
		Introduction of biochemical reactive group	
ManNLev	Human T-lymphoma (Jurkat) Neutrophil derived (HL-60) Cervical epithelial carcinoma (HeLa)	Ketone group expression on cell surface used for the selective chemical addition of ligands	(Mahal et al., 1997)
		Altering tumour cells sialylation	
ManNProp	Rat leukemic (RBL-3H3)	Altering Surface polySia for Immunotargeting tumour cells	(Liu et al., 2000)
ManNProp **ManNPent**	Human neuroblastoma (SH-SY5Y)	Reduced cell surface sialylation, migration and invasion	(Gnanapragassam et al., 2014)
ManNProp	Human neuroblastoma (IMR-32)	Reduced polysialylation and migration	(Seifert et al., 2012)

Keywords: ManNProp: *N*-propanoyl; ManNBut: *N*-Butanoyl; ManNPent: *N*-pentanoyl; ManNGc: *N*-glycolyl; ManNLev: *N*-levulinoyl; ManNPent (*N*-pentanoyl) mannosamine.

4.1.1 Aims and objectives

The main aims of this chapter are to evaluate potential novel inhibitors of polyST developed in-house at the ICT, in terms of their cytotoxicity, specificity and their effect on polySia expression and invasion.

The aims will be addressed by the following objectives:

- Assessment of novel polySTi cytotoxicity using the MTT assay (absence of cytotoxicity desirable)
- Confirmation of the specificity of the novel compounds for α-2,8-polySTs over other sialyltransferases (α-2,3-ST and α-2,6-ST) using differential labelling of sialic acids
- Investigation of the effects of novel compounds on modulation of cell surface polySia expression using the recovery assay characterised in Chapter 3
- Determination of the functional effect of the novel molecules on neuroblastoma and glioma cell invasion using a 2D Matrigel invasion assay optimised in Chapter 3

4.2 Materials and Methods

4.2.1 Materials

Unless stated, all reagents, enzymes and antibodies details are as described in Chapter 2 and Chapter 3.

4.2.2 Compounds

A series of novel polySTi synthesised at the ICT are listed in **Table 4.2**. Stock solutions of each compound were prepared in 100% DMSO at 100 mM and stored in aliquots at -20°C.

For the assays, the compounds were diluted down in culture media so that the highest concentration of DMSO was 0.1%.

4.2.2.1 Cell lines

Cell line details are given in Chapter 2. Two cell lines SH-SY5Y and C6-STX were selected for these studies as examples of high polyST expression, whilst C6-WT was used as a low expressing negative control. Unless specified, cells were cultured in their specified medium as described in section 2.2.3.1.

4.2.2.2 MTT assay

Cell cytotoxicity was assessed in polySia positive cells (IMR-32, SH-SY5Y, C6-STX) and polySia negative cells (C6-WT) using the MTT assay as described in section 2.2.3.3.

Cells were treated with the novel compounds at the following concentrations: 1, 5, 10, 50 and 100 µM, and microplates evaluated spectrophotometrically after 96 h to determine the long-term effect of the compounds on cells. Since for the invasion assay a 24 h time course was used, the MTT assay was also performed for 24 h to determine the short term effect of the novel polySTi. After 24 h media was aspirated and replaced with fresh medium and incubated and the plate was read after 96 h.

Table 4.2: Compounds evulated in this thesis.

***N*-acylmannosamine analogues**	**Rationally designed inhibitors**	**Tool compound**
ICT3149	ICT3125	CMP
ICT3128	ICT3126	
ICT3172		
ICT3176		

4.2.3 Determining the specificity of novel inhibitors for α-2,8-polyST

Compound specificity for α-2,8-polyST was characterised as specified in Chapter 2. Effects on other sialic acids were evaluated using *Maackia* amurensis isoforms and *Sambucus nigra,* which detect α-2,3 and α-2,6 sialic acids respectively. Cell cultures were treated with 100 µM of compound for 24 h and then administered as stated in section 2.2.6.

4.2.4 Evaluation of polySTi efficacy and specificity

4.2.4.1 PolySia knockdown assay

The effect of the novel compounds on polySia tumour cell surface expression was evaluated on polySia expressing cells SH-SY5Y and C6-STX using the recovery assay as defined in Chapter 3.

Briefly, cells were treated with endo-N for 24 h to remove polySia and cultures fixed directly to confirm removal of polySia. Cultures were treated with endo-N first and then incubated with medium (polySia recovery) or exposed to different concentrations of agent (50 µM and 100 µM) following 6 h incubation time. Then cultures were processed through the polySia recovery assay using CMP (cytidine monophosphate as tool compound) at 5 mM described in section 3.2.2.

4.2.4.2 Analysis of the effects of the novel compounds on polySia mediated cell invasion

The effect of novel agents on polySia mediated invasion was tested using the Matrigel invasion assay as described in Chapter 3. In the Matrigel invasion assay, Matrigel coated inserts were prepared by adding 0.1 ml of Matrigel solution (250 µg/ml) to the transwell and allowing the Matrigel to dry at 37°C in a non-humidified oven for 2 h. Cells were starved for 4 h in serum free medium. Trypsinized cells were suspended in serum free medium and cells were added to the upper chamber of transwells with medium or polySTi (100 µM). Medium with 10% FBS was added to the lower chamber. After 24 h, transwell were processed as described in section 3.2.3.1. Here, CMP at 5 mM was used as a positive control.

4.2.5 Statistical analysis

Each experiment was performed in triplicate. Results are presented as mean ± SD. Quantifiable data were subjected to the Student's *t*-test for statistical significance. Differences were considered statistically significant ($p<0.05$) as used in literature (Kiss et al, 1994; Joshi et al, 2005).

4.3 Results

4.3.1 Evaluation of cytotoxicity of novel compounds using the MTT assay

IMR-32, C6-STX, SH-SY5Y and C6-WT cells were exposed to novel compounds for 96 h (**Figure 4.2**). Minimal cytotoxicity was seen with the majority of the compounds evaluated as an IC_{50} was not achieved, even for the highest concentration used, 100 µM.

IC_{50} values were achieved for two compounds for 96 h exposure; ICT3176 and ICT3172 have IC_{50} less than 100 µM (**Table 4.3**). ICT3176 IC_{50} values were achieved in IMR-32 (90 µM) and C6-STX (90 µM), whilst for ICT3172 IC_{50} values were seen for in IMR-32 (50 µM), SH-SY5Y (30 µM) and C6-STX (40 µM) (**Table 4.3**).

The assays used in this thesis have compound exposure lasting for 24 h. Since cytotoxicity was clearly seen for 96 h exposures and this may result in an issue with longer exposure time in the assay. Therefore, SH-SY5Y, C6-STX and C6-WT cells were exposed to compounds for 24 h: no IC_{50} values were obtained from three independent experiments on SH-SY5Y, C6-STX and C6-WT cells, giving confidence that any inhibitory effects (i.e. reduction in polySia surface expression or cell invasion) of ICT compounds will be as a result of polyST inhibition, not compound cytotoxicity (**Figure 4.3**).

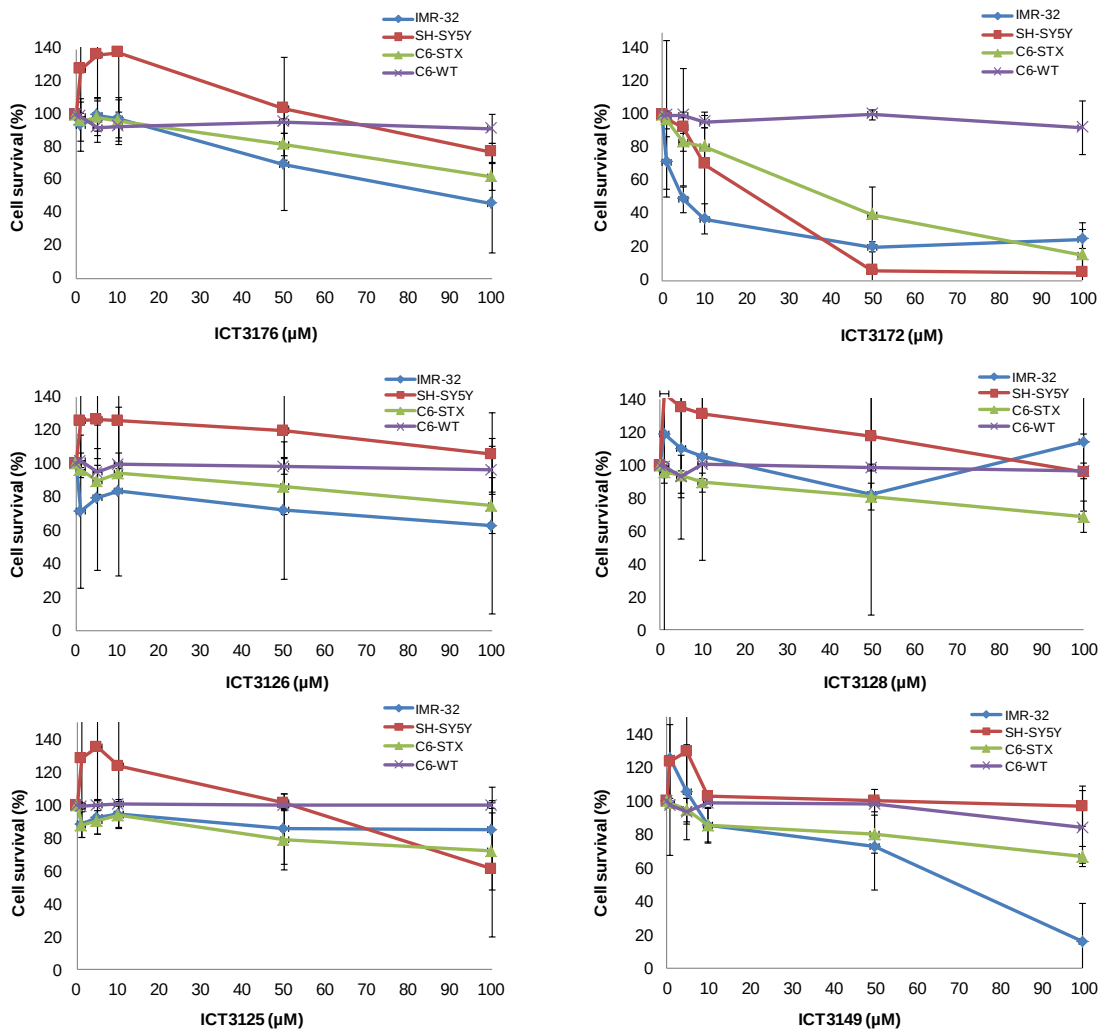


Figure 4.2: Evaluation of novel compound cytotoxicity (96 h).

IMR-32, SH-SY5Y, C6-STX and C6-WT cells were incubated with different concentrations of compound and were assessed using the MTT assay as described in the Materials and Methods. The results are expressed as percentage of cell survival (n=3).

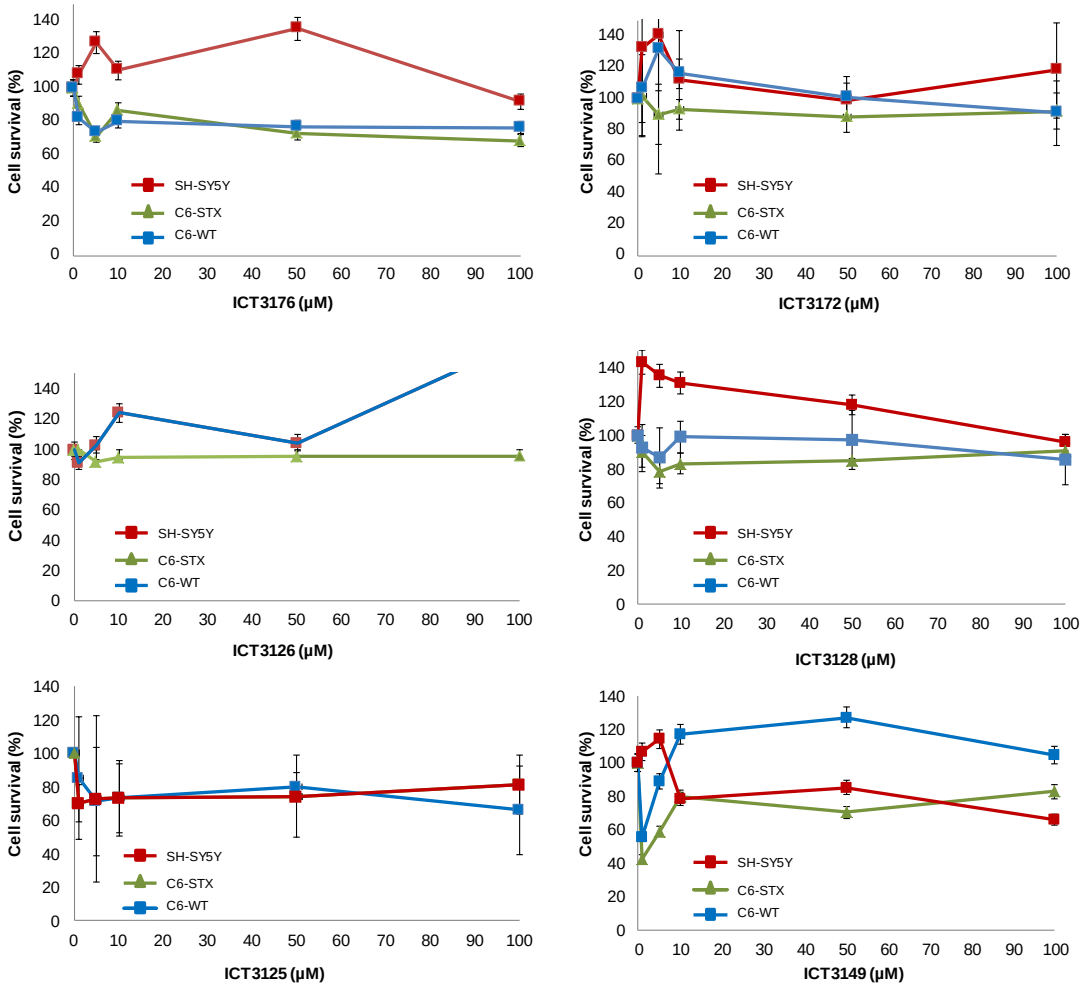


Figure 4.3: Evaluation of novel compound cytotoxicity (24 h).

A graphic presentation of SH-SY5Y, C6-STX and C6-WT cell lines were exposed to increasing concentration of six novel compounds. The results are expressed as percentage of cell survival.

Table 4.3: Cytotoxicity IC_{50} (IC_{50} ± SD) values of novel compounds towards tumour cells at 96 h incubation period as determined by the MTT assay (n=3).

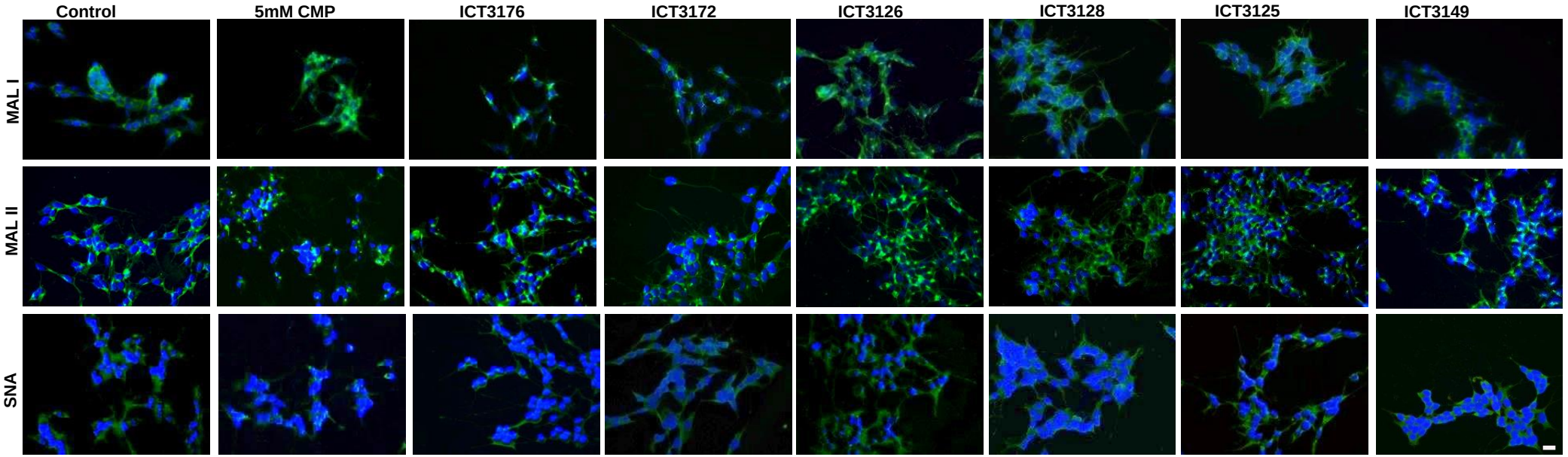
Novel compound	IMR-32	SH-SY5Y	C6-STX	C6-WT
ICT3176	90 µM ± 29.96	>100 µM	90 µM ± 8.35	>100 µM
ICT3172	50 µM ± 30.24	30 µM ± 5.85	40 µM ± 11.42	>100 µM
ICT3126	>100 µM	>100 µM	>100 µM	>100 µM
ICT3128	>100 µM	>100 µM	>100 µM	>100 µM
ICT3125	>100 µM	>100 µM	>100 µM	>100 µM
ICT3149	>100 µM	>100 µM	>100 µM	75 µM ± 26.69

4.3.2 Novel compounds demonstrate no effect on α-2,3 and α-2,6 sialic acid expression

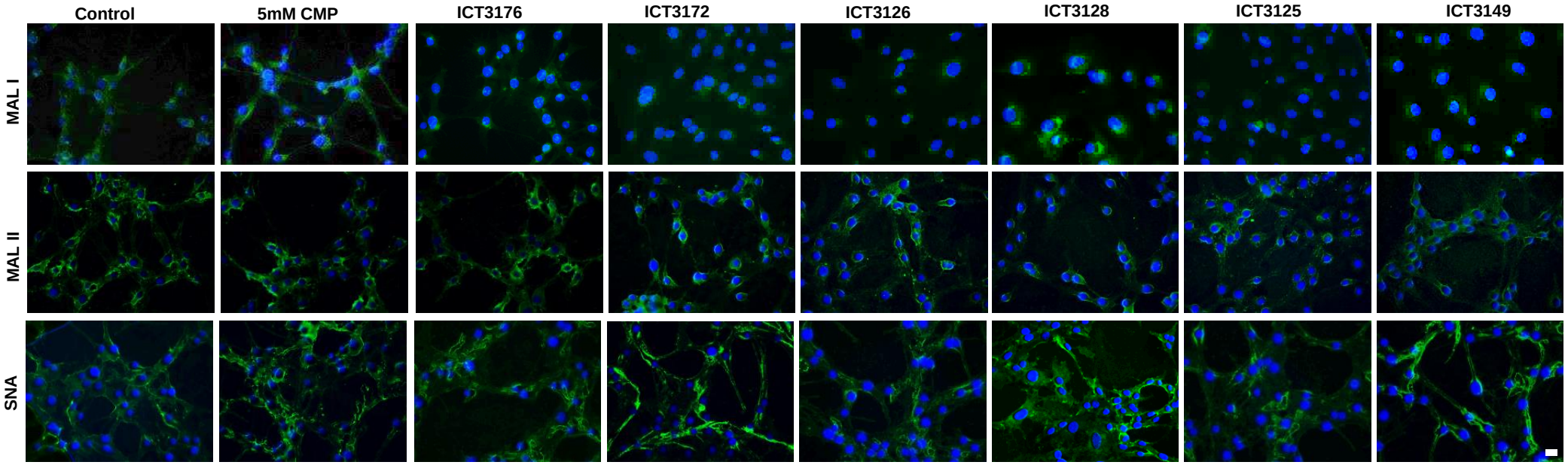
SH-SY5Y, C6-STX and C6-WT cells were also subjected to a staining procedure based on the sialic acid-specific lectins, SNA and MAL I and MAL II, which is specific for α-2,6-linked and the latter α-2,3-linked sialic acids shown in **Figure 4.4**. Six potential polySTi, namely: ICT3172, ICT3176, ICT3126, ICT3128, ICT3125 and ICT3149 were screened at a non-toxic concentration, 100 µM.

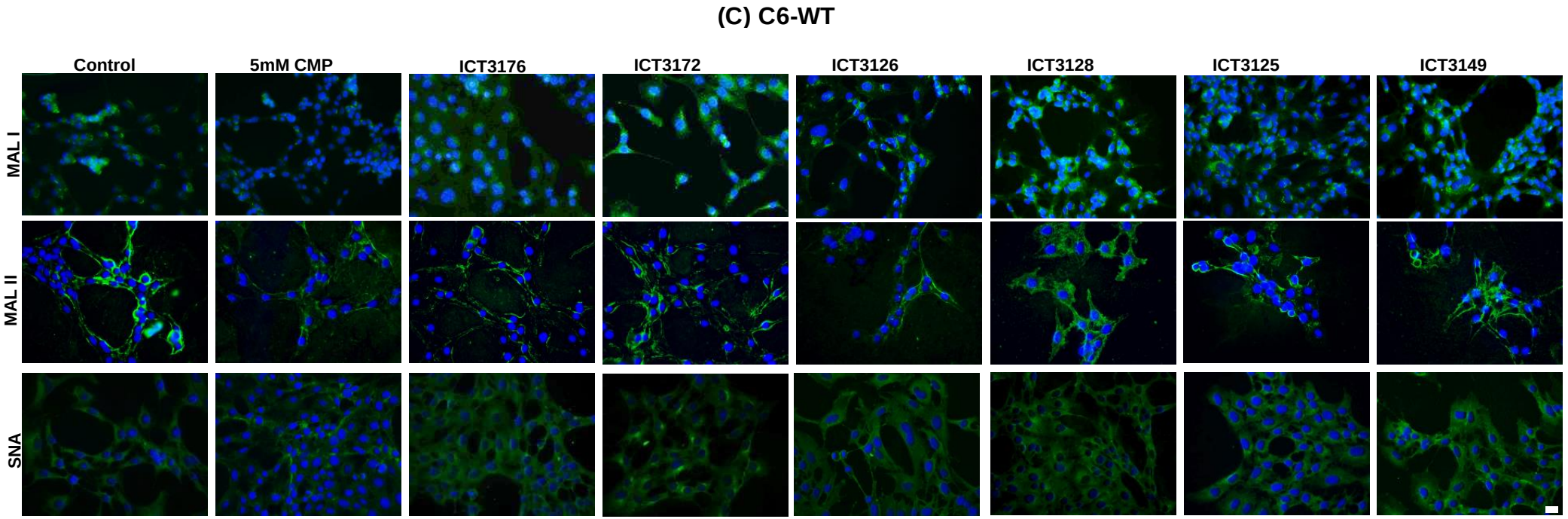
Treatment of potential polySTi had no significant effect on α-2,6-linked and α-2,3-linked sialic acid expression as judged by lectin staining specific for these Sias. This suggested that these inhibitors are specific for α-2,8-polyST as confirmed by the quantification of expression shown in **Figure 4.4D**.

(A) SH-SY5Y


Control
5mM CMP
ICT3176
ICT3172
ICT3126
ICT3128
ICT3125
ICT3149
MAL I
MAL II
SNA

(B) C6-STX


Control
5mM CMP
ICT3176
ICT3172
ICT3126
ICT3128
ICT3125
ICT3149
MAL I
MAL II
SNA


(C) C6-WT
Control
5mM CMP
ICT3176
ICT3172
ICT3126
ICT3128
ICT3125
ICT3149
MAL I
MAL II
SNA

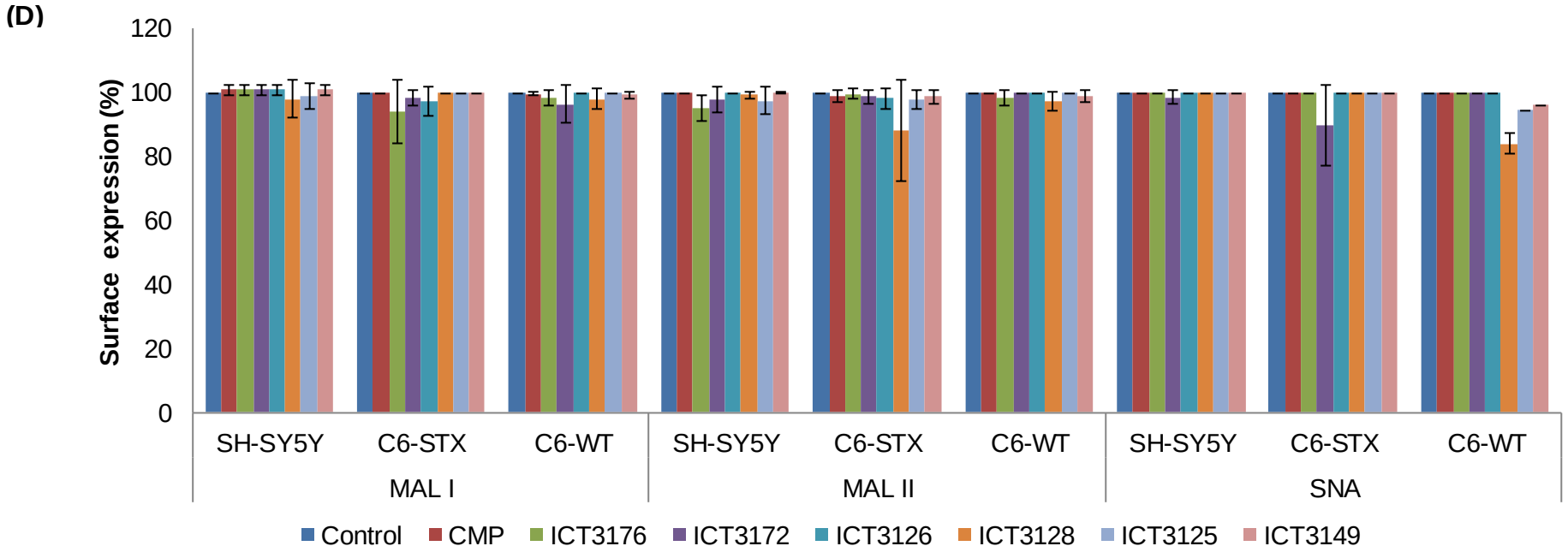


Figure 4.4: Effect of potential polySTi on expression of α-2,3 and α-2,6 sialic acids, as determined by lectin staining.

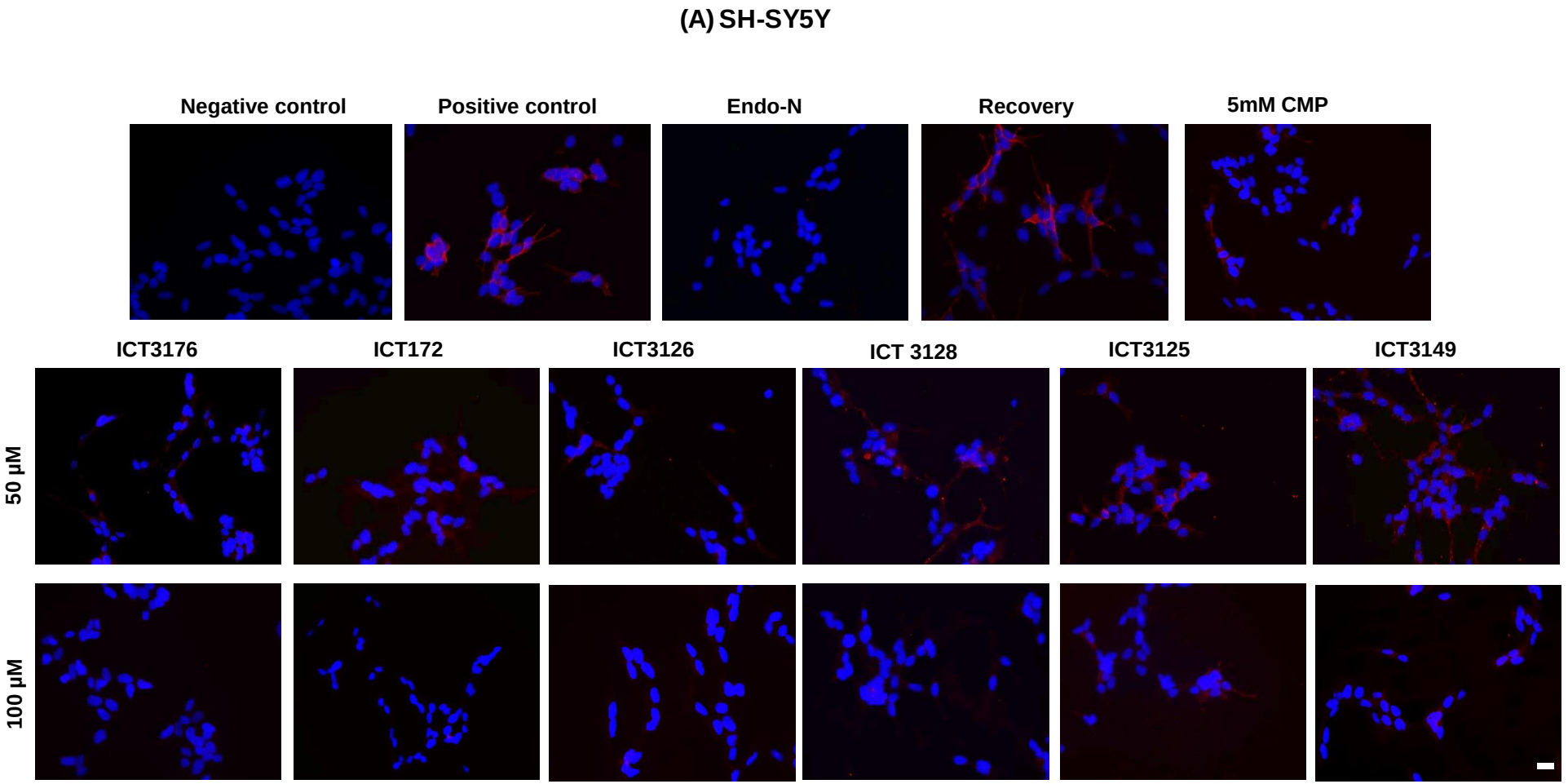
(A) SH-SY5Y, (B) C6-STX and (C) C6-WT cells were stained with *Maackia amurensis agglutinin* (MAL) in its two isoforms MAL I and MAL II to detect the expression of α-2,3-linked sialic acids and with *Sambucus nigra agglutinin* (SNA) to detect the expression of α-2,6 linked sialic acid. (D) Quantitative analysis of cells after 24 h polySTi treatment. There was no significant reduction seen in the expression of other sialic acids. Data is represented as mean ± SD of three independent experiments.

4.3.3 Evaluation of effects of novel agents on cellular polySia expression

Using the recovery assay developed in Chapter 3, the effects of novel compounds on modulation of polySia cell surface expression were evaluated.

First, cell surface polySia expression was removed by endo-N and then its re-expression was explored in the absence or presence of different potential polySTi. The non-toxic concentrations 50 and 100 μM (see **Figure 4.3**) were selected for 24 h treatment.

In untreated SH-SY5Y and C6-STX cells, tumour cell surface polySia expression had recovered in 6 h whereas cells treated with potential polySTi showed an absence of polySia (**Figure 4.5** and **Figure 4.6**). At the highest concentration, ICT3176 (~60%) and ICT3172 (~65%) markedly halted polySia re-expression in SH-SY5Y cells. Other novel polySTi were less effective in SH-SY5Y cells (**Figure 4.5**) at the concentrations tested. With C6-STX cells, similar results were seen. No cytotoxicity was seen confirming that the absence of polySia is likely to be to the inhibition of polyST. ICT3128 also affected polySia re-expression of C6-STX cells more than SH-SY5Y cells (**Figure 4.6**, $p<0.05$). ICT3176 was the most potent novel compound evaluated.


(A) SH-SY5Y
Negative control
Positive control
Endo-N
Recovery
5mM CMP
ICT3176
ICT172
ICT3126
ICT 3128
ICT3125
ICT3149
50 µM
100 µM

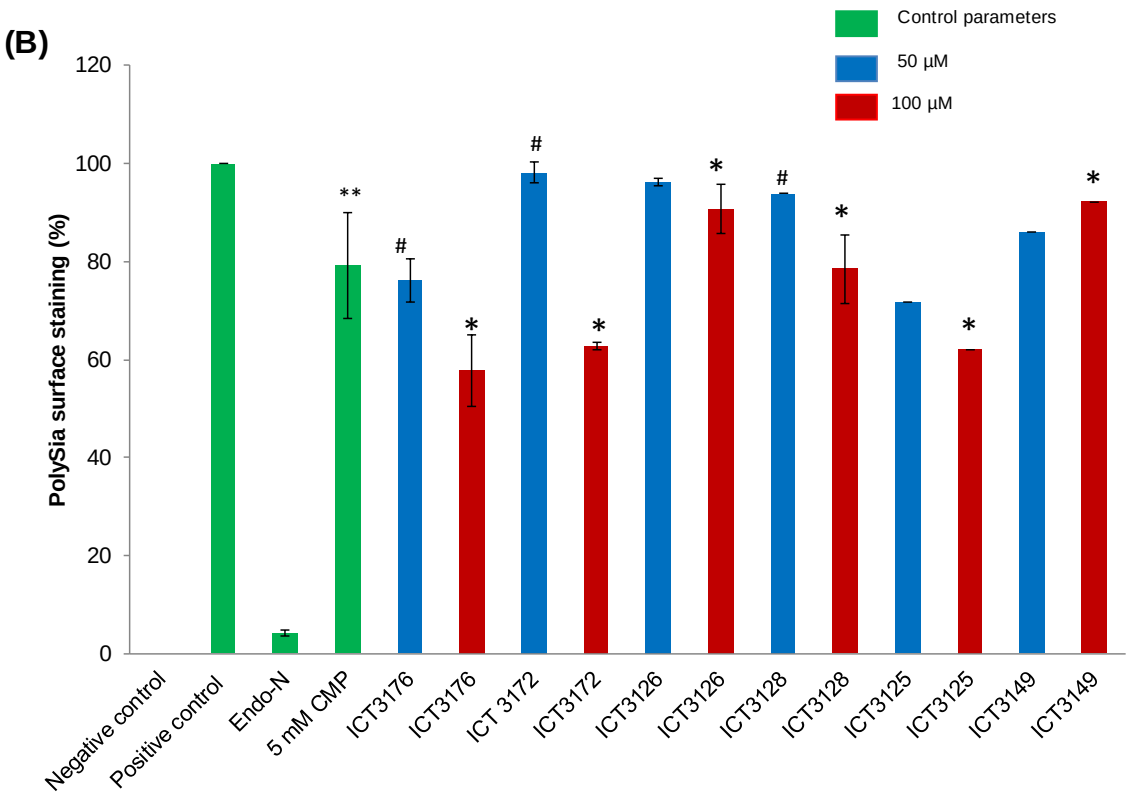
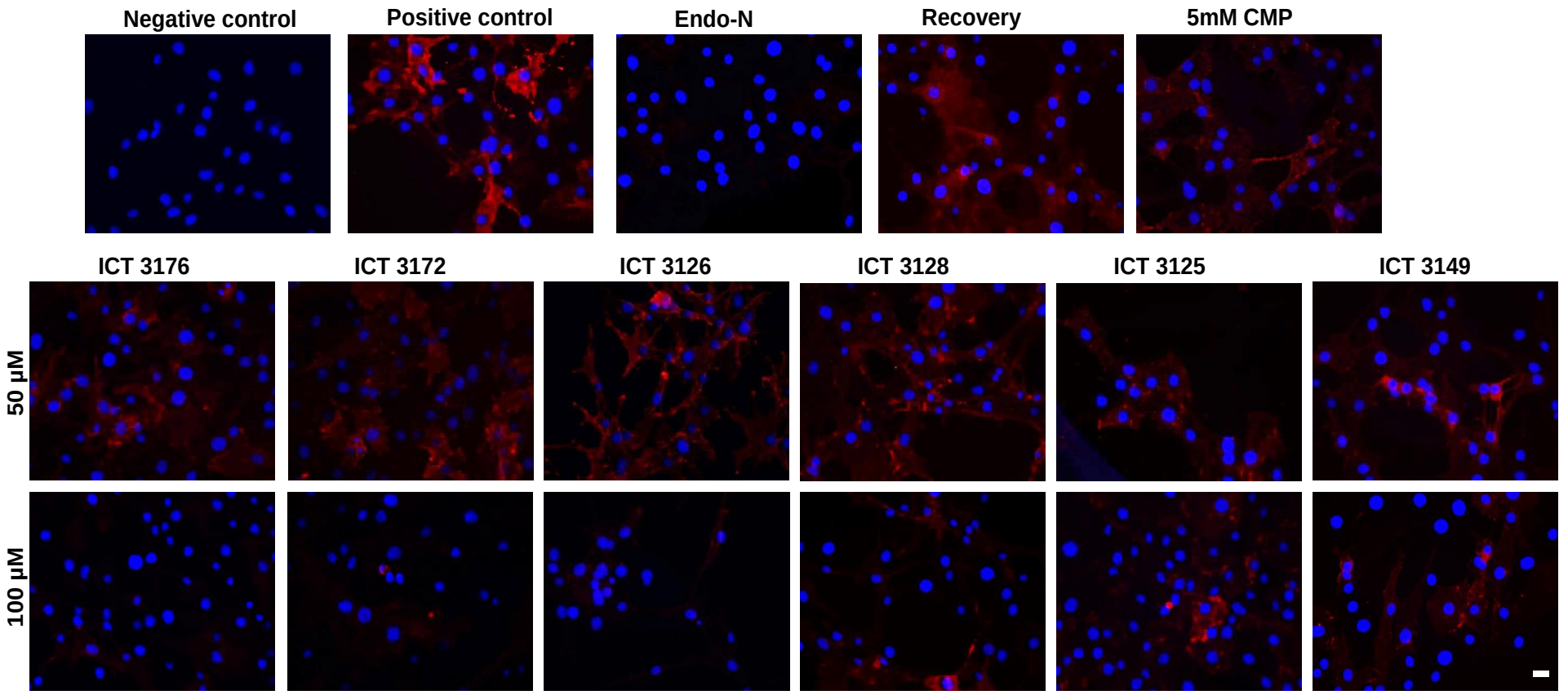


Figure 4.5: Effect of potential polySTi on polySia re-expression of SH-SY5Y cells using the polySia recovery assay.

(A) Cells were treated with endo-N for 24 h, rinsed and allowed to recover with medium or compound. Control data was not treated with endo-N. Quantification of SH-SY5Y cells represents the sum of polySia expression divided by sum of nucleus (DAPI) (B). Data was normalised to the result observed with untreated cells (positive control) and represents as mean ± SD. Statistically significant results were obtained with CMP (**$p<0.05$) and all ICT compounds tested at 100 µM, as compared with untreated cells (positive control) ($p<0.05$, indicated by *). ICT3176, ICT3172 and ICT3128 produced statistically significant inhibition at 50 µM ($p<0.05$, indicated by #).


(A) C6-STX
Negative control
Positive control
Endo-N
Recovery
5mM CMP
ICT 3176
ICT 3172
ICT 3126
ICT 3128
ICT 3125
ICT 3149
50 µM
100 µM

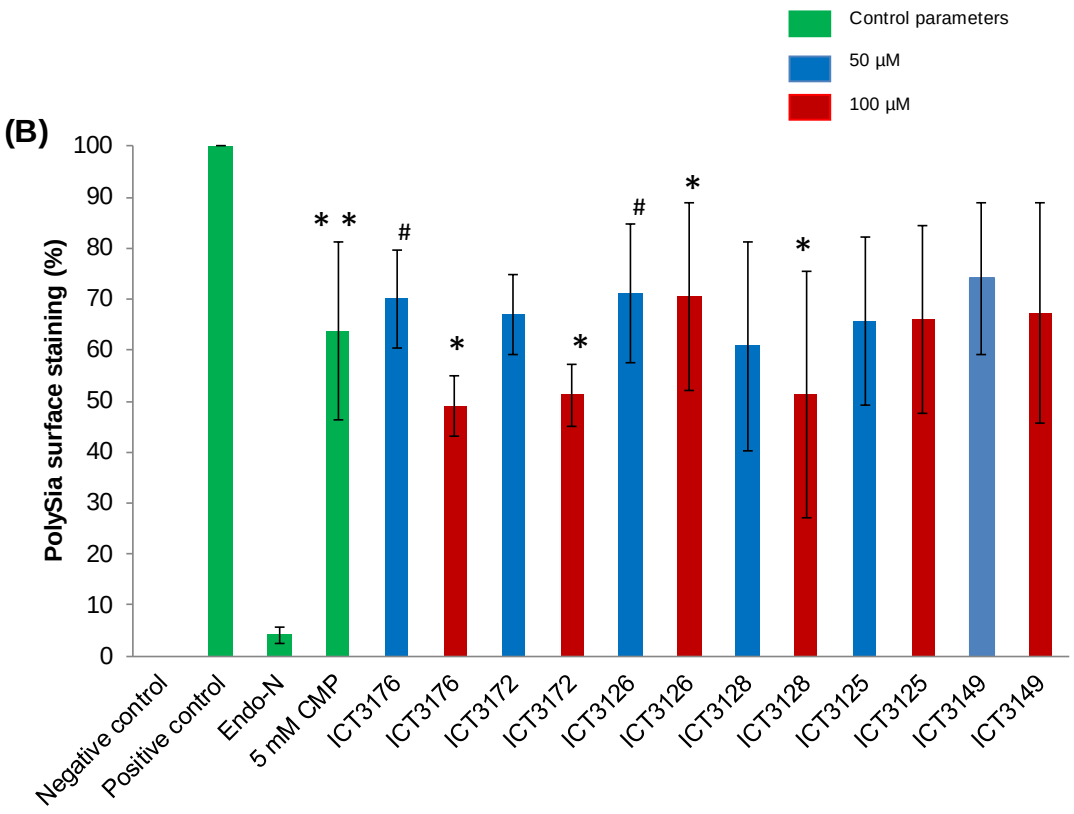


Figure 4.6: Effect of novel compounds on polySia re-expression of C6-STX cells using the polySia recovery assay.

C6-STX cells were treated with endo-N for 24 h, rinsed and allowed to recover with medium or novel compounds (A). Control data was not treated with endo-N. Quantification of C6-STX cells represents the sum of polySia expression divided by sum of nucleus (DAPI) (B). Data was normalised to the result observed with untreated cells (positive control) and represents as mean ± SD. Statistically significant results were obtained with ICT3176, ICT3172, ICT3128 and ICT3126 at 100 µM, as compared with untreated cells (positive control) ($p<0.05$, indicated by *). ICT3176, ICT3126 and ICT3172 produced statistically significant inhibition at 50 µM ($p<0.05$, indicated by #).

4.3.4 Novel compounds reduce SH-SY5Y and C6-STX invasion

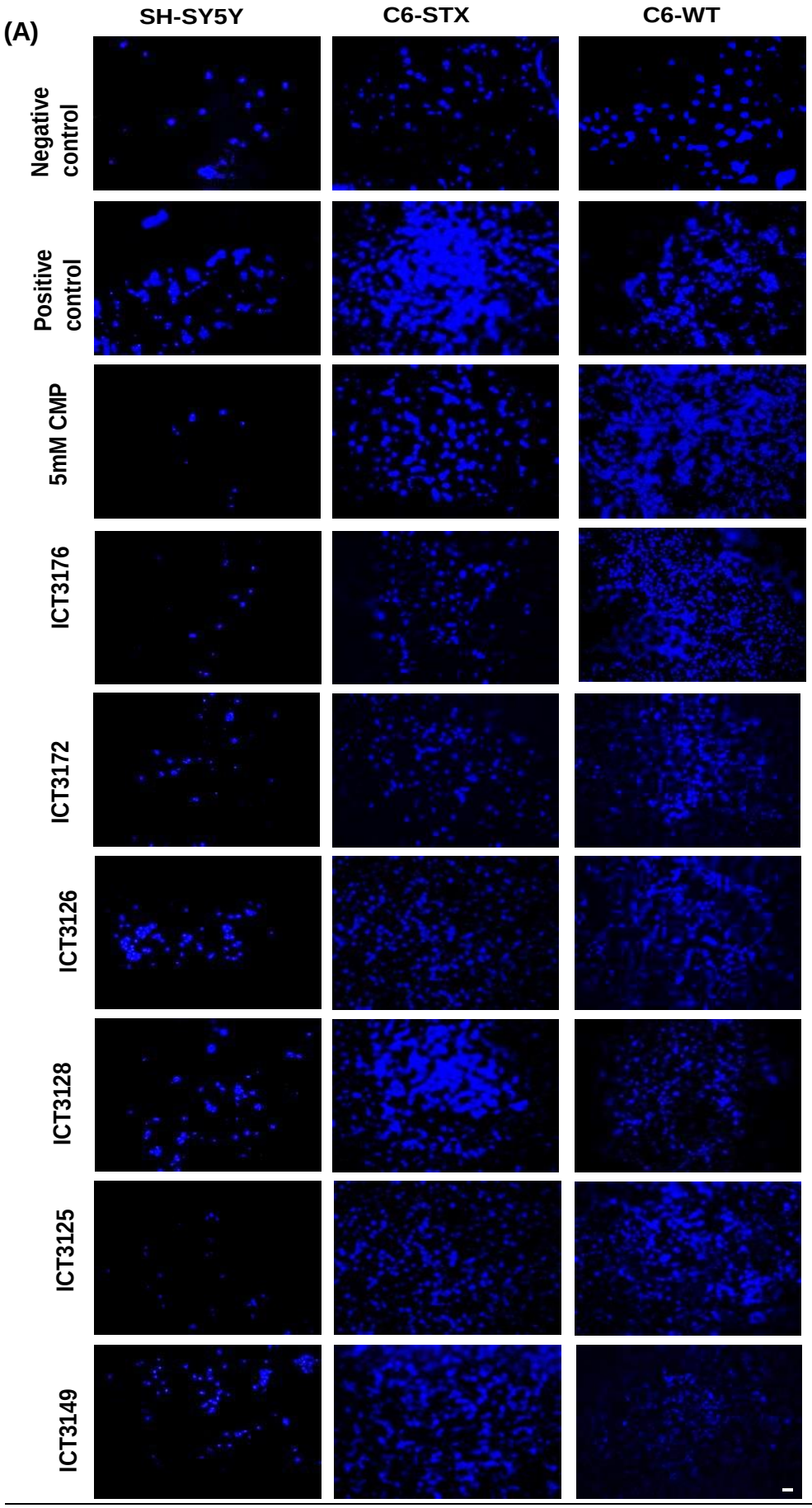
Next, the effect of novel polySTi on polySia dependent invasion was examined using the Matrigel invasion assay.

Cells were exposed to non-cytotoxic doses of polySTi, 100 µM (see **Figure 4.3**) for 24 h treatment.

As shown in **Figure 4.7**, the number of invading cells observed in seven random microscopic fields in the Matrigel invasion assay was also significantly reduced ($p<0.05$ in relation to untreated control) by ICT3176 and ICT3172 in SH-SY5Y and C6-STX cells at 100 µM.

ICT3149 showed a lower inhibitory effect in invasion in C6-STX and SH-SY5Y cells at the concentration tested. No effect of novel compounds was seen for C6-WT (polySia and polyST negative) cells invasion.

No cytotoxicity was observed in any of the experiments.


(A)
SH-SY5Y
C6-STX
C6-WT
Negative control
Positive control
5mM CMP
ICT3176
ICT3172
ICT3126
ICT3128
ICT3125
ICT3149

(B)

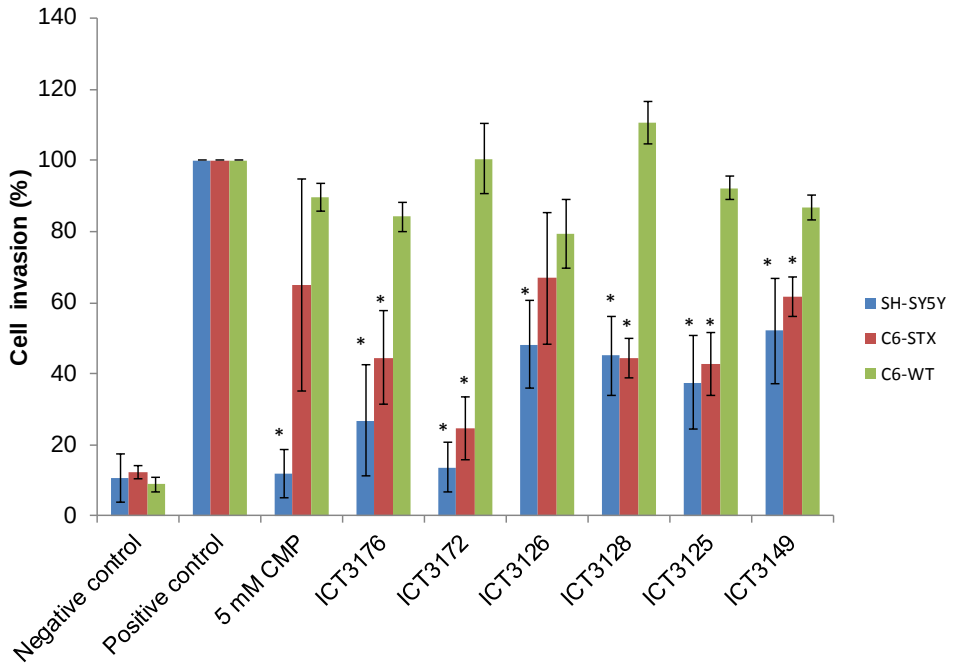


Figure 4.7: Evaluation of invasion of tumour cells following novel agents treatment.

SH-SY5Y, C6-STX and C6-WT cells incubated with novel compound (100 µM) for 24 h. Cells invading the lower surface of the Boyden chamber were stained with DAPI and images were captured under a microscope (magnification, X20) (A). Invaded cells were counted and normalized to the result observed in untreated cells where *$p<0.05$ is considered significant (B). All the data results are from three independent experiments and represented as mean ± SD. Scale bar: 50 µm.

4.4 Discussion

Many elegant studies have demonstrated the flexibility of the incorporation of unnatural Sia in the biosynthetic pathway of Sia (Charter et al., 2002, Liu et al., 2000, Mahal et al., 2001). More than twenty years ago, (Kayser et al., 1992) described the novel tool (synthetic *N*-acyl mannosamines; ManNProp) to characterise the biological functions of Sia.

Many studies have published data about ManNProp (acts as polySia pathway inhibitor) reducing migration in IMR-32 cells (Seifert et al., 2012) and reduction in cell surface polysialylation, invasion and migration in neuroblastoma SH-SY5Y cells (Gnanapragassam et al., 2014). These studies used high concentrations of ManNProp (up to 10 mM) in cell-based assays, including cell toxicity, proliferation, migration and invasion (Gnanapragassam et al., 2014, Seifert et al., 2012). Higher millimolar concentrations were required by these studies to inhibit or reduce polysialylation, due to the highly hydrophilic nature of molecules (they are sugars). These concentrations of Sia precursors are considerably higher than typically required for clinically-used drugs such as 5-fluorouracil or cisplatin (Gnanapragassam et al., 2014), but nevertheless these molecules are useful as molecular tools to study biological effects of polysialylation.

At the ICT, a series of *N*-acylmannosamine precursors were designed and synthesised with design features to aid entry of the compound into cells. These molecules were modified precursors (acetylated) of ManNProp, successfully

resulting in a reduction in the concentrations required to produce biological effects at micromolar levels. This was due to an increase in lipophilicity and thus membrane permeability, allowing these compounds to enter into cells more easily.

Preliminary screening of potential polySTi using the MTT assay showed an absence of toxicity with short term treatment (24 h) whereas slight toxicity was observed with a long term treatment (96 h) of ICT3172 and ICT3176 in IMR-32, SH-SY5Y and C6-STX cells. This cytotoxicity was observed previously: for example a study by Liu et al (2000) showed that leukaemia (RMA) cells were similarly affected by ManNProp. Therefore, the effects seen for the novel compound ICT3176 was most likely due to inhibition of polyST (leading to reduction of the polySia surface expression and as a consequence invasion) in polySia expressing SH-SY5Y and C6-STX cells, instead of due to the compound toxicity.

CMP, previously identified as a competitive polyST inhibitor (Al-Saraireh et al., 2013), was additionally evaluated. The CMP concentration used in this study showed no cytotoxic effect on these same cell lines. Since IMR-32 cells grow in clumps and thus do not form monolayers, they were not further utilised in the recovery and invasion assays.

The specificity of potential polySTi for α-2,8-polyST was confirmed, with no effect on α-2,3-ST or α-2,6-ST, as determined by absence of effect on the expression of α-2,3 (using MAL I, MAL II) or α-2,6 sialic acid (using SNA).

Therefore, the effect of novel agents was further investigated using the polySia recovery assay. The results indicated the different polySTi had differing effects on the recovery of polySia. Significant inhibition of polySia cell surface recovery was observed following ICT3176 and ICT3172 treatment in both polySia positive cells, whereas no effect was seen in C6-WT cells.

ICT3149 had the lowest effect on SH-SY5Y and C6-STX polySia cell-surface expression at the concentrations tested. This was predicted, since ICT3149 is a precursor to the natural substrate (acetylated ManNAc), and so effectively increases the concentration of Sia/polySia in cells. The slight inhibitory effects seen on invasion may be the result of altered cell-cell or cell-matrix adhesion, which result from increased polySia expression. This requires further investigation (Liu et al., 2000). ICT3128 is the acetylated version of ManNProp and had some reasonable effect on SH-SY5Y and C6-STX cells, at the lower (µM) concentrations previously described.

As shown in **Figure 4.5** and **Figure 4.6**, ICT3176 and ICT3172 produced the highest reduction in tumour cell surface polySia expression. These molecules are analogues of ICT3128, in which the propyl group has been substituted for an alkyl and aromatic substituent respectively. These agents emerged as the most promising from a wider panel of ManNProp derivatives.

In the polySia recovery assay, anti-polySia antibody (mAb 735) was used, which is specific to native polySia (Frosch et al., 1985). Using other antibodies, such as monoclonal anti-*N*-propanoyl neuraminic acid antibody (mAb 13D9), could be useful to assess the effect of certain polySTi on polySia biosynthesis.

MAb 13D9 was generated against meningitis and (details in **Table 2.1**) specifically recognises extended epitopes (e.g. *N*-propionyl-PolySia, Neu5Prop, which could result from ManNprop treatment) (Pon et al., 1997), since mAb 735 does not recognise polySia exclusively with elongated *N*-acyl chains (Liu et al., 2000). Both antibodies have been used together previously to study *N*-acyl sialic acid precursor treatment (Pon et al., 2007). The specificities of these antibodies would allow us to investigate the incorporation of unnatural Sia precursors into polySia chains and alternation on polySia cell surface to its analogue required further investigation.

Interestingly, acetylated *N*-acylmannosamine-based polyST inhibitors reduced the invasion ability of the SH-SY5Y and C6-STX cells, which express a significant amount polySia and STX. Different novel polySTi did show similar effects in the recovery assay.

As shown in **Figure 4.7**, ICT3149, ICT3125, ICT3128, ICT3126, at 100 µM were not as effective in inhibiting SH-SY5Y and C6-STX invasion compared to ICT3172 and ICT3176. These effects suggest that these particular acetylated molecules possess more desirable characteristics in terms of their *N*-acyl side-chains, to achieve polyST inhibition. This was in agreement with effects observed previously by the Horstkorte group, where ManNProp was shown to be able to interfere with polysialylation, but only in STX expressing cells (Horstkorte et al., 2004a).

Furthermore, this data is consistent with earlier studies which have used ManNProp, which demonstrates that the down-regulation of polysialylation is a

useful tool to control tumour migration and invasion (Gnanapragassam et al., 2014, Seifert et al., 2012). Thus, ICT3176 and ICT3172 were the most functionally effective inhibitors of polyST, with no cytotoxic effects observed on the concentrations used in the cells studies here.

4.5 Conclusion

In this study, six novel potential inhibitors of polyST were screened in the polySia recovery and invasion assays. Two compounds ICT3176 and ICT3172, demonstrated a significant reduction in tumour cell surface polySia expression and invasion of SH-SY5Y and C6-STX cells. Specificity of polySTi for α-2,8-polyST over other STs was confirmed by lack of effect on α-2,3 and α-2,6 sialic acids expression.

Acetylated *N*-acylmannosamines are useful tools to study the effects of polyST inhibition on cell function. Although these molecules are hydrophilic, they possess sufficient lipophilicity to be used at micromolar concentrations. Further investigation of these interesting molecules is warranted to determine whether they possess the desired characteristics to be evaluated *in vivo*.

CHAPTER FIVE

5 Discussion and Future perspective

5.1 General discussion

In recent decades, there have been significant developments in the understanding of the clinical biology of neuroblastoma. This has led to current therapeutic approaches used for high-risk patients, which incorporate induction chemotherapy (high dose chemotherapy), local treatment with surgical resection and radiotherapy, and maintenance therapy 13-*cis*-RA and immunotherapy, as reviewed in Chapter 1. Despite these options, many neuroblastoma patients still have a poor prognosis (Brodeur, 2003). Furthermore, these drugs have had limited success caused by complications including high tone deafness (Pearson et al., 1992), secondary cancer (Kushner et al., 1998), infection and acute toxicity (including bone pain and allergic reaction) (Modak and Cheung, 2010). Due to these limiting toxicities, the

introduction of further cytotoxic chemotherapy is not considered feasible (Ishola and Chung, 2007).

Despite some success with these treatments it is estimated that 50% of high-risk patients suffer relapse (Maris, 2010) and hence new approaches are being explored to treat this ailment. The biggest challenge is to bridge the gap between understanding the neuroblastoma biology and developing effective therapies. The predominant hallmark of neuroblastoma is metastasis. Prevention of tumour progression by targeting dissemination could be a powerful approach to treating patients, especially in high-risk neuroblastoma patients.

One option for the timing of therapeutic intervention by an agent lacking cytotoxicity to target metastasis in neuroblastoma, would be after initial treatment to remove the primary tumour through surgery, when minimal residual disease (MRD) is a concern. Such a therapy would provide a good opportunity to manage this disease (Weinstein et al., 2003, Matthay et al., 1999). Indeed this is the period when other novel agents have been administered. For example, anti-GD2 (ganglioside) antibody therapy and 13-*cis*-RA have been shown to be effective in the MRD setting (Reynolds, 2004, Yu et al., 2010). The presence of neuroblastoma cells in peripheral blood circulation could be monitored to evaluate the effects of therapy (e.g. using STX, polySia or GD2 mRNA as a surrogate marker) (Reynolds, 2004), with an expectation of reduction of circulating cells with efficacy of adjuvant therapies (Cheung et al., 2003).

Despite these new therapies, neuroblastoma recurrence from MRD after treatment remains a significant problem. One possibility is that MRD is thought to result from tumour-initiating cells which are drug resistant (Hartomo et al., 2013). Another possibility is the failure of the immune system to recognise neuroblastoma cells. This is possibly due to gangliosides and sialic acids (important for metastasis) expressed on tumour cells enabling evasion of the immune system (Cheung and Dyer, 2013, Potapenko et al., 2007).

Targeting specific pathways and associated molecules could provide insights into developing biologically-based therapies to control MRD in high-risk neuroblastoma patients. For example, retinoids are natural and synthetic derivatives of vitamin A, known to induce neuroblastoma cell differentiation *in vitro* (Sidell et al., 1983). These include all trans-retinoic acid (ATRA), 13-*cis*-RA and fenretinide (4-HPR) (Reynolds et al., 2003). The first clinical trial carried out by Matthay et al (1999) showed 13-*cis*-RA to induce apoptosis (rather than differentiation) and increase survival in high-risk neuroblastoma patients, following bone marrow transplantation (Matthay et al., 1999, Weinstein et al., 2003). Indeed, 13-*cis*-RA is now part of the standard treatment protocol for high-risk neuroblastoma in an effort to reduce MRD (Brodeur and Bagatell, 2014, Matthay et al., 2009). Fenretinide (a synthetic retinoid) might have activity for MRD by inducing apoptosis in neuroblastoma cells and is currently in paediatric Phase I studies (Villablanca et al., 2011).

Other agents including crizotinib, which target cells with changes in anaplastic lymphoma kinase (*ALK*), a mutation found in 15% of neuroblastoma patients (Sahu et al., 2013). This may block cancer cell proliferation by inhibiting

processes associated with tumour growth and dissemination. Crizotinib is currently subject of on-going clinical trials (ClinicalTrials.gov, 2015b, Mossé et al., 2013, Krytska et al., 2015).

Other compound with potential to treat disseminated high-risk neuroblastoma patients include vorinostat (a histone deacetylase inhibitor), currently undergoing Phase II trials (DuBois et al., 2015). Vorinostat is a small molecule which inhibits tumour cell growth by repressing expression of genes (such as vascular endothelial growth factor receptor) that are required for growth. The agent has shown synergy with 13-*cis*-RA, which is believed to sensitise tumour cells to the drug (ClinicalTrials.gov, 2015a, Ramalingam et al., 2007). Patients in Phase I studies showed a complete response to therapy when vorinostat was administered with 13-*cis*-RA in MRD (Fouladi et al., 2010). Phase II studies are now underway.

There is strong evidence that neuroblastoma metastatic behaviour is associated with components of the tumour glycocalyx (Berois and Osinaga, 2014). Investigations are underway to incorporate immunotherapy targeting surface molecules in treating high-risk neuroblastoma patients with MRD. The approach already being taken using antibodies targeting ganglioside GD2, a sialic acid-containing glycosphingolipid, which is overexpressed in neuroblastoma and other neuroendocrine tumours (Mujoo et al., 1987). Since GD2 has tumour-selective expression and as its expression is on the cell surface, this makes it an attractive anti-cancer immunotherapy, since anti-GD2 antibodies can mediate neuroblastoma cell lysis by two mechanisms including complement-

dependent cytotoxicity (CDC) and antibody-dependent cellular cytotoxicity (ADCC) (Mujoo et al., 1987).

Immunotherapy is now part of mainstream neuroblastoma therapy, with Unituxin (the tradename for the GD2-binding monoclonal antibody) having been approved this year by the Food and Drug Administration (FDA) and the European Medicines Agency (EMA) (FDA, 2015, EMA, 2015). Two types of anti-GD2 antibody (chimeric (ch)14.18 and mouse 3F8) were tested in clinical trials extensively. The combination of 3F8 with granulocyte-macrophage colony stimulating factor (GM-CSF) and 13-*cis*-RA showed an 80% overall survival rate after first remission in high-risk patients (Cheung et al., 2012). Ch14.18 administered with interleukin-2, GM-CSF and 13-*cis*-RA was also proven to be effective in randomised trails (Yu et al., 2010). These studies suggested a significant improvement with combinational therapy in high-risk neuroblastoma patients, in the MRD setting. The clinical trials additionally showed the approach to be safe in children (Kushner et al., 2001, Ozkaynak et al., 2000). The therapy does have issues. However, side effects including severe pain, fever, urticaria and allergic reactions (pruritus) are all common (Navid et al., 2010).

As has been reviewed in this thesis, polySia is a key component of the tumour glycocalyx, which may equally be amenable to therapeutic intervention. The MRD period of treatment provides an opportunity for novel approaches such as a polySTi to prevent further metastatic spread. Given that polySia could also protect tumour cells from the immune system (Drake et al., 2008), there is potential for long-term use as maintenance therapy for high-risk neuroblastoma

patients. This novel approach could result in significantly better outcomes for high-risk neuroblastoma patients.

The expression of polysialylated NCAM correlates with high metastatic potential and poor prognosis in numerous malignant tumours, including neuroblastoma (as reviewed in Chapter 1). Although the precise roles of polySia in neuroblastoma tumorigenesis are still being uncovered, it has been postulated to be associated with a highly tumorigenic subpopulation of cancer stem cells or tumour-initiating cells in another polySia-expressing tumour, namely glioma, where these cells promote tumour angiogenesis and resistance of cells to therapy (Amoureux et al., 2010, Bao et al., 2008).

Another possibility is that NCAM polysialylation promotes tumour cell dissemination. This is supported by various *in vitro* and *in vivo* studies. *In vitro*, the presence of polySia promotes migration (Eggers et al., 2011, Suzuki et al., 2005). *In vivo* xenograft studies in neuroblastoma have demonstrated that polySia-expressing tumours (LAN-1, LAN-5) demonstrate evidence of disseminated micrometastases, something not observed from polySia-NCAM-negative cell lines (Kelly, SK-N-SH) (Valentiner et al., 2011). Scheidegger et al (1994) established two sublines E2 (no polySia expression) and E3 (with polySia expression) from a human SCLC-derived cell line NCI-H69. High metastatic potential was seen with the E3 clone compared to the E2 clone. Daniel et al (2001) described a model of human rhabdomyosarcoma, cell line TE671, which expresses polySia and NCAM. Lung metastases were observed following intravenous, intramuscular, and intraperitoneal, but not subcutaneous (s.c) injection of TE671 cells into nude mice. Repeated injections of endo-N (an

enzyme which cleaves polySia from NCAM) led to decrease in polySia expression in primary intraperitoneal nodules and ascites; and diminished formation of liver or lung metastases. This, along with similar studies, provides strong evidence for the association between polySia NCAM expression with tumour invasion and metastasis (Suzuki et al., 2005, Daniel et al., 2001, Daniel et al., 2000).

In a series of published studies, it has been suggested that it is possible to manipulate NCAM polysialylation regulated by STX through treating cells with unnatural Sia precursors, *N*-acylmannosamines, including ManNProp, resulting in low or abrogated expression of polySia (Mahal et al., 2001, Liu et al., 2000, Horstkorte et al., 2004a, Charter et al., 2002). For example, the expression and function of the polyST enzymes was studied by Horstkorte et al (2004a), who showed that treating human embryonic carcinoma cell line (N-T2) cells with ManNProp resulted in loss of polySia.

Evidence was provided that STX was selectively inhibited by the respective unnatural *N*-acyl sialoside, which is metabolically formed from the respective precursor *N*-acyl mannosamine. These molecules can therefore be considered as useful chemical tools, with selectivity for STX over PST. This is significant since PST (as opposed to STX) is important for the limited polySia expression found in the brain post-embryogenesis, which is required for neuronal plasticity (Ong et al., 1998).

With this increasing evidence of polyST inhibition as a therapeutic strategy, the ICT has established a programme to identify small molecule inhibitors of

polyST. As part of this programme, this project has set out to establish assays to assess novel molecules, with some promising and encouraging findings in the polySia recovery and invasion assays.

The use of ManNProp and similar compounds presents as a significant challenge in *in vitro* and *in vivo* studies. Since these are polar molecules, they are poorly suited to crossing lipid membranes. Therefore high (mM) concentrations of ManNprop have been used in earlier studies to maximise the amount of compound crossing the cell membrane since no known transport mechanisms have been identified (Büttner et al., 2002).

Novel *N*-acylmannosamines have been synthesised at the ICT, with features which should allow the molecules to better cross biological membranes, therefore requiring lower concentrations. For example: 5 to 10 mM concentration of ManNprop (ICT3147) is required to be effective in the scratch assay (in-house data), whereas analogue ICT3176 is shown to be effective at 100 µM (Chapter 4). This strategy reduces the concentration by a factor of 50-100 and has been successfully utilised to modulate polySia expression on NCAM.

Recent studies (Seifert et al., 2012, Gnanapragassam et al., 2014) have demonstrated that treatment of neuroblastoma cell lines (IMR-32 and SH-SY5Y) with ManNprop reduced polySia surface expression, migration and invasion *in vitro*, suggesting that the concept of polyST inhibition has potential for neuroblastoma therapy. Similar results were found here with polySia expressing neuroblastoma cells (SH-SY5Y) treated with novel agents at 100 µM.

The most effective compounds from the panel were ICT3176 and ICT3172, which showed significant reductions in polySia tumour surface expression and invasion. These compounds possess lipophilic *N*-acyl side chains, which suggest that the *N*-acyl position of the respective sialic acid may be important for polyST inhibitory activity. Other compounds studied proved less potent: ICT3128, and ICT3149 each required higher concentrations for activity (see Chapter 4). ICT3125 and ICT3126, potential inhibitors identified from virtual screening of drug-like structures also proved less potent. However, these compounds serve as interesting starting points for future medicinal chemistry.

Given these encouraging results, a preclinical strategy is required to evaluate novel structural iterations of the promising molecules and to progress molecules towards the clinical studies. **Figure 5.1** shows a typical example of a 'screening cascade'. The biological assays specific to a target of interest, in this case is polyST will be utilised here.

At the ICT, small molecules have been designed and synthesised. Then, a cell free assay is employed to quantify the polysialylation using a fluorescent acceptor, a 1,2-diamino-4,5-methylenedioxy-benzene (DMB)-labelled trimer of α-2,8-linked sialic acid (DMB–DP3), which acts as an NCAM substitute and primes polyST. The inhibitory activity of potential compounds against polyST is determined by quantification of the main product (in this case DMP-DP4) as described by Al-Saraireh (2013).

Next, cytotoxicity was determined using the MTT assay and compounds demonstrating little or no toxicity were progressed further, since it would be

expected that polySTi should not be toxic to cells. Selected compounds were then evaluated in the polySia recovery assay (Chapter 3 and Chapter 4). With this assay, the indirect effect of compounds on polySTi was determined by inhibiting the recovery of polySia in the presence of polySTi. Compounds were then selected for evaluation in assays assessing facets of the tumour dissemination process (i.e. adhesion, migration, and invasion).

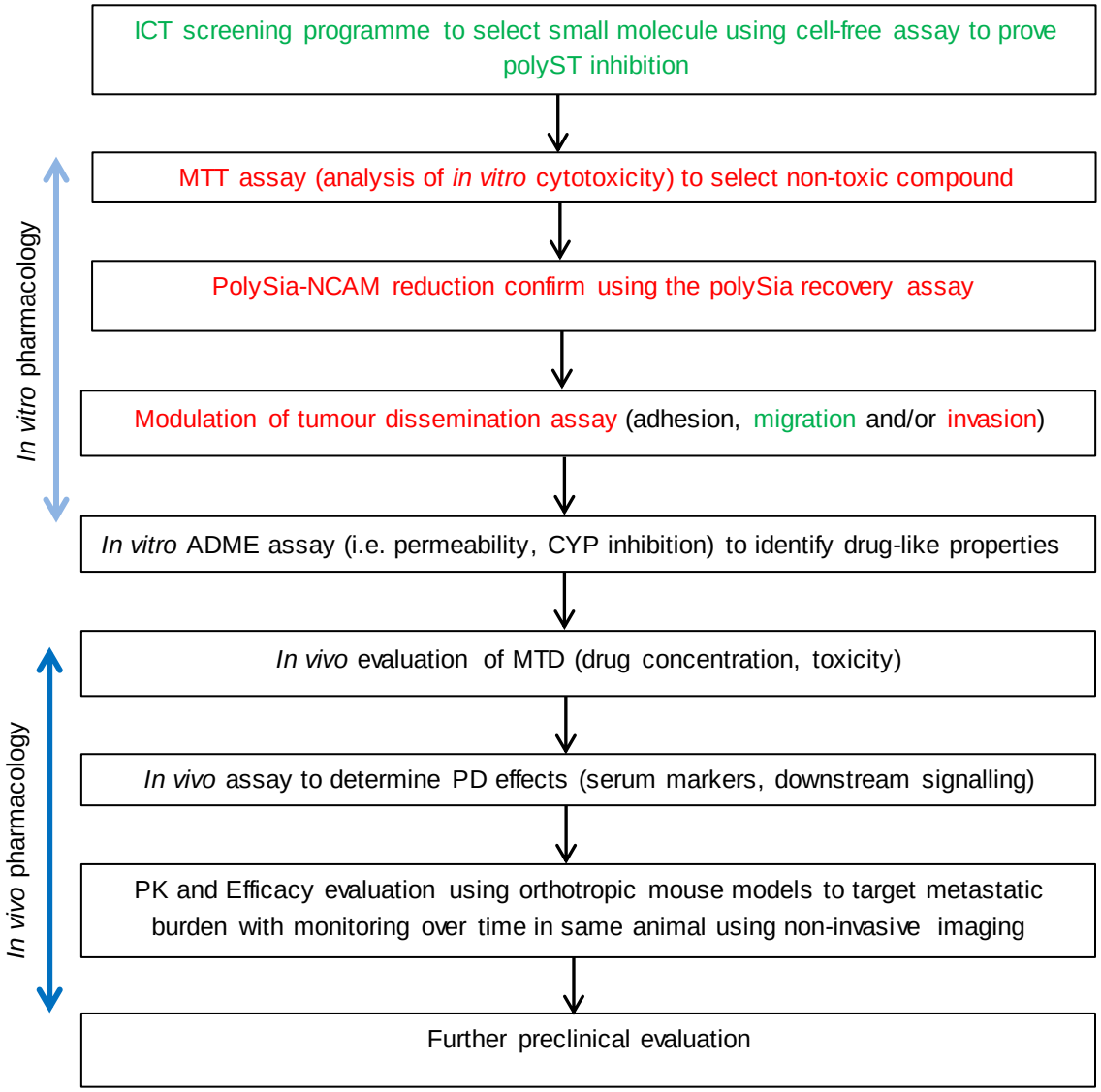


Figure 5.1: An example of a representative screening cascade could be potential used for identify small molecule polySTi.

The first step is to identify potential polySTi using a cell-free assay. Then, the MTT assay and the recovery assay confirmed non-toxic reduction/inhibition of polySia cell surface expression. Progressing down the screening cascade, reduction in polysialylation NCAM analysed using *in vitro* tumour dissemination assay. Compounds with reasonable activity and ADME features will process to *in vivo* assay to determine maximum dose, toxicity, and PD profile. (Green: in-house data; red: this thesis data; black: future work).

Keywords: MTD: Maximum tolerance dose; ADME: absorption, distribution, metabolism and excretion; CYP: Cytochrome P450; PD: Pharmacodynamics; PK: Pharmacokinetics.

As has been discussed, metastasis is a multi-faceted process, including invasion and migration of tumour cells. Inhibiting neuroblastoma cell invasion would provide therapeutic benefit for neuroblastoma patients. Tumour cell invasion was the focus of this study, primarily using a Matrigel-based transwell assay (Chapter 3). Future work for novel compound effects in other facets of the metastatic process will also need to be examined. Assays including those studying migration and adhesion are described in section 5.2.

Once a compound has shown activity in the above assays (i.e. reduction of polySia-NCAM expression and functional evidence of reduction of the metastatic process under evaluation), there is a need to consider the potential issues with metabolism before processing to next stage, *in vivo* screening. Various assays would focus on assessing the potential compound liabilities and it is important to predict appropriate features of compounds with respect of ADME (absorption, distribution, metabolism and excretion). A cytochrome P450 (CYP) assay could predict toxic and cellular metabolic effects caused by novel compounds as CYPs are key enzymes in the liver involved in drug metabolism (Hoelder et al., 2012). The stability of the drug could be determined using liver microsomes/hepatocytes (containing metabolising enzymes) which would allow us to predict compound clearance (Li, 2005) and potential toxicity (Hughes and Karlén, 2014). Solubility and permeability are important factors to determine the potential of a compound to be drug-like enough to be tested. A stable and soluble compound could potentially be administered systemically or orally.

In the case of this thesis, the novel polySTi ICT3176 is a carbohydrate probe. It has some water solubility and has been modified by acetate groups to increase

hydrophobicity (thereby enhancing membrane permeability). These changes may affect the pharmacokinetics of this compound sufficiently to allow *in vivo* administration (Ernst and Magnani, 2009). This could be the subject of future studies.

ICT3125, identified via virtual screening, is a more drug-like compound (it is not a carbohydrate). The molecule does contain carboxyl group, which will limit its cell permeability, possibly resulting in a lack of potency (Chapter 4). Therefore, the structure of this compound needs further modification to improve its lipophilicity and effectiveness.

Once the desired *in vitro* parameters are achieved, the compound can then progress to *in vivo* evaluation as suggested in **Figure 5.1**. For *in vivo* studies, the selection of the dose level and dosing frequency (drug, stability, amount to be given and formulation) is based on the drug maximum tolerated dose (MTD) and any dose-limiting toxicities (DLT) (Carter, 1987). The bioavailability of the compound, as determined in pharmacokinetic studies, is also important.

With regard to *in vivo* studies, a few questions would need to be considered in future studies. For example, a method to enable measurement of the effect of polyST inhibition would be important, since standard effects on primary tumour growth inhibition may be limited. Therefore, pharmacodynamics (PD) assays using a biomarker would be useful. Previously it has been mentioned that the expression of polysialylated NCAM has been measured in serum, and that this correlates with tumour expression in neuroblastoma (Gluer et al., 1998d). This could thus be a useful PD marker. Another strategy could be to identify and

evaluate a downstream signalling pathway with effects known to be due to modulation of polySia on NCAM, as discussed in section 1.6.4.

In the design of any experiments using animals it is important that 3Rs (Reduction, Refinement and Replacement) principles are adhered to. This principle was first proposed by Russell and Burch (1959), who stated that every effort should be made to ***R***eplace animal usage with non-sentient alternatives, or if animals need to be used in experiments, to ***R***educe the number of animals to a minimum and to ***R***efine experimental procedures so minimum pain and distress is caused (Russell and Burch, 1959, Flecknell, 2002). If alternative methods could be applied to address the experimental hypothesis e.g. cell culture, use of lower organisms human tissue, then these should be considered instead of the standard in vivo experiments (Doke and Dhawale, 2015).

The choice of an appropriate *in vivo* model is crucial. For evaluation of effects on primary tumours then standard s.c transplanted neuroblastoma xenograft tumours in immunodeficient mice could be utilised. As an example, evaluation of fenretinide in combination with other agents (cisplatin, and vincristine) was carried out in SH-SY5Y neuroblastoma xenograft models (in CB17 s.c female mice) (Norris et al., 2014).

In another study, a liver metastases model was developed by injecting tumour cells into liver and has also attempted to reconstruct severe combined immunodeficiency (SCID) mouse immunity with human killer cells. Using this model, anti-GD2 antibody (ch14.18) with interleukin-2 was shown to be more effective against neuroblastoma (Sabzevari et al., 1994).

Given that the main therapeutic aim with a potential polySTi would be to limit tumour dissemination, then a xenograft model may not be ideal, since s.c tumours don't often metastasize in a manner that reflects the clinical setting (Hoffman, 1999). Orthotopic models of neuroblastoma which are metastatic and more clinically relevant would thus be a better model, as shown by a study in 2002 (Khanna et al., 2002). This model is generated by injecting tumour cells into the adrenal gland, where 40% of neuroblastoma arises (Maris et al., 2007, Teitz et al., 2011). An orthotropic mouse model, using SH-SY5Y neuroblastoma cells also implanted in the adrenal gland, was used to examine *in vivo* sensitivity of bortezomib (proteasome inhibitor that blocks cell growth and angiogenesis in neuroblastoma) in combination with fenretinide (Pagnan et al., 2009). The combination of bortezomib and fenretinide acts through activation of endoplasmic reticulum (ER) stress genes, resulting in activating apoptosis and blocking angiogenesis. This pharmacological combination proved promising as a potential neuroblastoma therapy (Pagnan et al., 2009).

Another study showed the delivery of nanoparticles encapsulating miR-34a (pro-apoptotic microRNA) conjugated with a GD2 antibody into a neuroblastoma orthotopic xenograft model (NB1691 or SK-N-AS cells implanted behind the left adrenal gland), and showed tumour specific delivery resulting in apoptosis and reducing tumour growth (Tivnan et al., 2012). Importantly, these studies enabled evaluation of both tumour growth and metastasis, in a manner which is more clinically relevant to the disease than an s.c xenograft.

Using these models, the effect of polySTi on metastatic burden could be evaluated. Non-invasive imaging methods would be useful for monitoring

tumour burden over time, in the same animal following treatment, with clear benefits in terms of 3Rs (see above). Examples of non-invasive imaging techniques which could be used include magnetic resonance imaging (MRI), computed tomography (CT) and bioluminescence. The potential utility of bioluminescence in not only to detect neuroblastoma tumours at an early stage, but also monitoring disease course, has been demonstrated in a murine model of neuroblastoma reported by (Dickson et al., 2007).

5.2 Future directions

Future studies will aim to select and progress polySTi towards clinical development, using a screening cascade similar to that described above. The experimental models described in this study are simple in *vitro* and *in vivo* models that allow us to determine the initial effect of potential polySTi on polySia tumour expression and facets of the dissemination process. As a result of work completed in this thesis, it would be important to perform further studies in the short-term to strengthen the promising findings seen. Hence, the immediate future of further *in vitro* evaluation will be focused upon in this section, with *in vivo* models already discussed in previous section. In the immediate future, the particular challenges include quantitative analysis of polySTi using flow cytometry and effect of potential polySTi on other aspects of tumour dissemination (migration and adhesion). PolyST knockdown experiments would be useful to confirm the effects observed with inhibitors.

5.2.1 Confirmation of the effects of potential polySTi using polyST gene knock-outs

Evidence for the effects of potential polySTi have been clearly shown using the polySia knockdown assay, in which endo-N removed polySia expression with re-expression being monitored in the presence of polySTi. As a further evidence of selectivity, small interfering RNA (siRNA) knockdown of the polyST enzymes, particularly STX (which is more associated with polySia expression in tumours than PST) could be utilised for further validation. This would result in more specific direct polyST knock down compared to the indirect polySia knock down

with endo-N. This approach has been successfully applied while investigating the role of polySia in pancreatic cancer. Results demonstrated that the siRNA knock-down of polyST resulted in an absence of polySia expression, and an associated reduction in tumour cell migration (Schreiber et al., 2008). Absence of any biological effect on such cells following treatment with a polySTi would thus confirm selectivity of the compounds.

5.2.2 Quantitative analysis of polySia expression following polySTi treatment

Two polySia labelling tools have been used, which have different merits. The first, mAb 735, is suitable for use in immunofluorescence experiments described in this study. The second, endoN-GFP, has been used in flow cytometry experiments previously to determine the effect of a known polySTi (CMP) in-house (Al-Saraireh et al., 2013). The work of this thesis presented semi-quantitative analysis of detecting polySia expression in a panel of tumour cells using the immunofluorescence technique. The main issue with this technique is the quality of fluorescent images, in which too much non-specific antibody or a higher concentration leads to misjudgement of the locations of molecules (Odell and Cook, 2013).

Alternatively, flow cytometry is a powerful tool, which can give us qualitative and quantitative data analysis of other molecules and cytoplasmic components (Brown and Wittwer, 2000). For example, the effect of Sia precursors (ManNProp, ManNAc and ManNPent) on SH-SY5Y cell surface polySia was examined using flow cytometry. The results showed 90% reduction in the

amount of polySia following treatment with ManNProp and ManNPent. This study shows how the effects of inhibitors on modulating polysialylation can be quantified using flow cytometry.

A previous study has indicated that polySia antibodies have different immunospecificity for degree of polymerisation and sialic acid species since the minimum chain lengths required for recognition are different (Sato et al., 2000). It will be interesting in future work to determine the immunospecificity of other anti-polySia antibodies. This is important in determining the effect of polySTi on polySia chain length to polySia biological roles using different anti-polySia antibodies. Also, the immunostaining of antibodies may be affected by the number of polySia chains on NCAM and immunostaining does not give the details of polySia structural features (i.e. number of polySia chains per NCAM) (Nishimura et al., 2014).

Therefore, combination analysis of biochemical methods (i.e. Western blotting) and chemical methods (i.e. high pressure liquid chromatography) could help us to further understanding of the modification or inhibition of polySia chains by polySTi.

5.2.3 Effect of potential polySTi on polySia mediated migration

In this thesis, tumour cell invasion was the primary focus. Other facets of the metastatic process would additionally need to be addressed, including migration and adhesion. The scratch assay is a very common method used at the ICT and has been utilised to show the inhibitory effect of CMP and other polySTi on

polySia-associated tumour cell migration ((Al-Saraireh et al., 2013); in-house data). This involves cells being seeded into multi-well plates, allowing cells to attach, forming a confluent monolayer. A needle or pipette tip is used to make a scratch forming cell-free zone and cells around the edges of the scratch can migrate (Yarrow et al., 2004). The scratch assay has also been employed in other studies showing that loss of polySia reduced migration and increased focal adhesion of tumour cells (Eggers et al., 2011).

Another commonly used assay in migration is the transwell migration assay, which has the similar technical setup mechanism as transwell invasion assay. The only difference is that transwell is overlaid by a thin layer of ECM (i.e. Matrigel) before seeding the cells in the chamber (Albini et al., 2004, Marshall, 2011). Both the transwell migration and invasion assay have been successfully used to show the effect of 13-*cis*-RA on migration and invasion of neuroblastoma cell lines (IMR-32 and SH-SY5Y) (Joshi et al., 2005). Therefore, different *in vitro* assays can be used to assess the effect of polySTi on polySia expressing migration.

5.2.4 Evaluation of different ECMs as substrate on polySia mediated adhesion

In previous work and also in this thesis, assays have been developed to look at two aspects of tumour dissemination. Another aspect, adhesion, has still not been fully addressed and is involved in tumour dissemination.

Interestingly, Beecken et al (2005) showed that modulating polysialylated NCAM resulted in blocking adhesion of several tumour derived cells (i.e. neuroblastoma cells UKF-NB-2, UKF-NB-3, and UKF-NB-4) to human umbilical vein endothelial cells (HUVEC). Five days administrations of VPA additionally delayed growth of neuroblastoma cells (Beecken et al., 2005).

Another study conducted by Li et al (2011) examined cells expressing polySia in terms of migration and invasion on different ECMs (i.e. Matrigel, collagen, and heparin). It was demonstrated that polySia activates FGFR and its downstream signalling components, PLC-γ, focal adhesion kinase (FAK) and extracellular signal-regulated kinase (ERK). The migration of fibroblast (NIH-3T3) cells transfected with STX (NIH-3T3-STX) and mock NIH-3T3 cells (NIH-3T3-mock) proved similar on different ECMs (fibronectin, VM and heparin). In each case, the polySia-expressing cells exhibited higher rates of migration. Effects on cell adhesion proved more complex, however.

Effects of polySia expression on cell adhesion varied according to the ECM studied: heparin (high), vibronectin, laminin and collagen IV (moderate) and fibronectin (poor). The author concluded that polySia promote cell migration, but that this does not strictly depend on its adhesion to different ECM substrates (Li et al., 2011). A cell adhesion assay has also been used with fibronectin coating, showing that ManNProp treatment affects calcium transport into the ER lumen and was thought to interfere with cellular signalling cascades (Horstkorte et al., 2004b).

As described in Chapter 1, polySia inhibits binding of NCAM and modulates tumour cell adhesion, migration and invasion. Therefore, polysialylated NCAM or downregulation of NCAM facilitates the migration of tumour cells from the primary site (Blaheta et al., 2006). Further investigation into the effect of novel polyST inhibitors on the ability of tumour cells to adhere to the extracellular matrix may provide a greater understanding of the role of polySia in the tumour cell dissemination process.

5.3 General conclusion

The inability to control tumour metastasis and subsequent cancer mortality is a major challenge. PolyST is a novel and unexplored therapeutic target, which plays a key role in neuroblastoma dissemination. The work presented in this thesis supports the hypothesis that a polySTi has potential to limit metastatic spread.

Cell-based assays for cell-surface polysialylation and tumour cell invasion have been developed and validated, enabling the identification of novel small molecule inhibitors of polyST. This study is a first presentation of drug-like polySTi having an effect on polySia cell surface expression and invasion. Also, this study for the first time demonstrates the selectivity of polySTi for α-2,8-polyST using a series of three sialic acid binding lectins. ICT3176 was the most promising inhibitor identified, with others (e.g. ICT3125) showing potential as providing a platform for medicinal chemistry development.

These results support the targeting of polyST as a therapeutic strategy for neuroblastoma and warrant further medicinal chemistry and *in vitro* and *in vivo* pharmacological investigations to identify a lead polySTi which could ultimately progress to the clinic.

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APPENDIX

Publications associated with this work

Contributions to peer reviewed publications

- Grant, C.; Twigg, P.; Saeed, R.F.; Lawson, G.; Falconer, R.A.; Shnyder, S.D. “Effect of polysialic acid expression on glioma cell nanomechanics.” Submitted to *BioNanoScience*.

Contributions to scientific conference abstracts

1. Falconer, R.A.; Ribeiro Morais, G.; Elkashef, S.M.; Viprey, V.; Saeed, R.F.; Springett, B.R.; Sutherland, M.; Loadman, P.M.; Shnyder, S.D.; Patterson, L.H. “Inhibition of polysialyltransferase modulates neuroblastoma cell migration and invasion.” YCR Annual Scientific Meeting, 2 July **2015**, Harrogate, UK. Abstract 7.

2. Elkashef, S.M.; Viprey, V.; Saeed, R.F.; Springett, B.R.; Sutherland, M.; Loadman, P.M.; Patterson, L.H.; Shnyder, S.D.; Falconer, R.A. "Polysialyltransferase ST8SiaII: a novel target for the treatment of neuroblastoma" AACR Annual Meeting, 18-22 April **2015**, Philadelphia PA, USA. Abstract 5431.

3. Viprey, V.; Saeed, R.; Springett, B.R.; Elkashef, S.; Sutherland, M.; Loadman, P.M.; Patterson, L.H.; Shnyder, S.D.; Falconer, R.A. "In vitro evaluation of inhibitors of polysialyltransferase ST8SiaII: a new target for neuroblastoma." BACR Development of Cancer Medicines – From Target to Disease: Modelling Stratified Approaches in Oncology, 27 November **2014**, Royal Society of Medicine, London. Abstract 27.

4. Falconer, R.A.; Viprey, V.; Springett, B.R.; Al-Saraireh, Y.M.J.; Northrop, M.; Sutherland, M.; Saeed, R.; Loadman, P.M.; Shnyder, S.D.; Patterson, L.H. "Modulation of neuroblastoma polysialic acid biosynthesis inhibits tumour cell migration and invasion." YCR Annual Scientific Meeting, 25 June **2014**, Harrogate, UK. Abstract 1.

5. Viprey, V.; Springett, B.R.; Al-Saraireh, Y.M.J.; Sutherland, M.; Northrop, M.; Saeed, R.; Loadman, P.M.; Patterson, L.H.; Shnyder, S.D.; Falconer, R.A. "Polysialyltransferase ST8SiaII: a new target for the treatment of metastatic tumours." AACR Annual Meeting, 5-9 April **2014**, San Diego CA, USA. Abstract 1774.

6. Viprey, V.; Springett, B.R.; Northrop, M.; Sutherland, M.; Saeed, R.; Loadman, P.M.; Cullinane, C.J.; Stahlschmidt, J.; Patterson, L.H.; Shnyder, S.D.; Falconer, R.A. "Polysialyltransferase ST8SiaII: a new target for the treatment of neuroblastoma." Neuroblastoma Research Symposium, 6-7 November **2013**, Liverpool, UK. Abstract 29.

7. Falconer, R.A.; Springett, B.R.; Viprey, V.; Sutherland, M.; Al-Saraireh, Y.M.J.; Saeed, R.; Begouin, M.; Northrop, M.; Loadman, P.M.; Shnyder, S.D.; Patterson, L.H. "Modulation of cell surface polysialic acid biosynthesis

inhibits tumour cell migration" YCR Annual Scientific Meeting, 26 June **2013**, Harrogate, UK. Abstract 9.

8. Springett, B.R.; Al-Saraireh, Y.M.J.; Viprey, V.; Sutherland, M.; Begouin, M.; Northrop, M.; Saeed, R.; Loadman, P.M.; Patterson, L.H.; Shnyder, S.D.; Falconer, R.A. "Inhibition of cell surface polysialic acid biosynthesis modulates tumor cell migration." AACR Annual Meeting, 6-10 April **2013**, Washington DC, USA. Abstract 4410.

9. Falconer, R.A.; Springett, B.R.; Al-Saraireh, Y.M.J.; Saeed, R., Shnyder, S.D.; Patterson, L.H. "Modulation of cell-surface polysialic acid expression and tumour migration" YCR Annual Scientific Meeting, 19 June **2012**, Harrogate, UK. Abstract 9.